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DATA SHEET

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PREFACE

This PhD project was carried out at the National Food Institute, Division of Toxicology and Risk Assessment, as well as the Department of Systems Biology, Enzyme and Protein Chemistry at the Technical University of Denmark under the supervision of Charlotte B. Madsen and Vibeke Barkholt. The project was funded by FOOD Denmark Research School, Danish Dairy Research Foundation and the Technical University of Denmark.

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Finally, I would like to thank my wonderful family, for support and patient, especially during the last period of the project.

SUMMARY

Food allergy is a major health problem in the Western countries, affecting 3-8% of the population. What makes a dietary protein a food allergen has not yet been established, though several characteristics have been proposed to be shared by food allergens. One of the features believed to be a general characteristic of food allergens is resistance to digestion. This is based on studies showing that allergenic dietary proteins in general are more resistant to digestion than dietary proteins with no proven allergenicity, concluding that a correlation between stability to digestion and allergenic potential exist. Resistance to digestion is for this reason a test parameter included in the safety assessment of the allergenic potential of novel proteins in genetically modified foods. The association between resistance to digestion and allergenic potential has though been challenged in recent years.

This PhD project aimed to investigate the sensitising potential of digestion products from the peanut allergen Ara h 1 and the cow's milk allergen *β*-lactoglobulin (BLG) in a Brown Norway (BN) rat model. Further the project aimed to compare the IgE binding epitopes of intact and digested Ara h 1.

This was done by digesting Ara h 1 and BLG in an *in vitro* model simulating the human gastric or gastro-duodenal digestion process. Simulated gastric digestion was performed with immobilised pepsin for 120 min at pH 2.5, while simulated duodenal digestion was performed with immobilised trypsin and chymotrypsin for 15 min at pH 6.5. Fractions of digestion products were made by separating the peptide fragments according to sizes in gel permeation chromatography (GPC). The intact allergens as well as digestion products hereof were thoroughly characterised by reverse phase high-performance liquid chromatography, MALDI-TOF mass spectrometry, amino acid analysis and GPC. To study the sensitising capacity groups of BN rats were immunised with the intact allergen or digestion products hereof by i.p. immunisation and specific antibody responses were examined by ELISAs, RBL-assay or avidity measurements. Comparison of intact and digested Ara h 1-specific IgE binding epitopes were performed by competitive immunoscreening using a random phage-displayed peptide library followed by mapping the identified IgE-binding epitope mimics on the surface of the Ara h 1 molecule. In addition to sera from the sensitised BN rats, sera from peanut allergic patients were used.

Both the gastric as well as the gastro-duodenal digests of the peanut allergen Ara h 1 were found to be very efficient for sensitising the BN rats. While gastric digest consisted of peptide fragments of up to M_r 4,000 the duodenal digest consisted of peptide fragments of up to M_r 2,000, yet both the peptide fragments in the gastric as well as in the gastro-duodenal digests were aggregated to complexes of larger sizes. After separation of the digested Ara h 1 into fractions the sensitising capacity was lost, though the IgE-binding capacity was retained. Epitope mapping of intact and digested Ara h 1 showed IgE binding epitopes of Ara h 1 to be conformational in origin and at least to some extent surviving the digestion process. For the peanut allergic patients five motifs were found to account for more than 65% of all identified epitope mimics and were found for both the intact as well as the digested Ara h 1. Digested BLG with peptide sizes of up to M_r 4,500 could on the other hand not induce any sensitisation response in the BN rats. They were instead suggested to possess tolerogenic capacity when co-administered together with intact BLG.

The results presented in the current thesis demonstrate that even very small peptide fragments, originally thought to be too small to act as a food allergens may indeed possess all features of a 'complete' allergen. This implies that an association between allergenicity and resistance to digestion is not an absolute feature of food allergens. The presented work indicates that peptide fragments may either possess sensitising capacity per se or that the observed allergenic capacity could be a result of the small peptide fragments aggregating to complexes of larger sizes. The importance of formation of aggregates is suggested by the epitope mapping study, where survival of conformational epitopes is demonstrated. This together with the

findings, that fractionation of digestion products leads to a loss of the sensitising potential, reveals that the allergenicity had to be more than simply a result of the small peptide fragments aggregating, and more a result of them being in an aggregated state resembling the intact Ara h 1 molecule. While small peptide fragments derived from one food allergen may retain sensitising capacity this is not necessarily the truth for other food allergens. This was demonstrated with the cow's milk allergen BLG, from which peptide fragments were shown not to be efficient for inducing any specific antibodies. Instead the results indicated that the peptide fragments derived from BLG had tolerogenic capacity, demonstrating that while some mixtures of peptides may guide the immune system in one direction, other mixtures of peptides may guide the immune system in another direction. Together these results demonstrate that several characteristics of digestion products from food allergens may collectively contribute the allergenic potential, where more than just peptide sizes and structures may contribute.

In conclusion, the experimental data presented in this PhD thesis contribute to the understanding of induction of allergy by investigating the sensitising potential of peptides derived from a food allergen. It add knowledge to our understanding of the mechanisms underlying the sensitisation, but at the same time points to the difficulties, if not infeasibilities, in identifying features that can be used as an ubiquitous marker for allergenicity of a dietary protein.

RESUMÉ (DANSK)

Fødevareallergi vurderes at være et voksende problem i de vestlige lande, hvor prævalensen ligger på 3-8% af befolkningen. Selv om vi spiser tusindvis af forskellige fødevareproteiner hver dag, er det kun et fåtal af disse der forårsager IgE-medieret allergi. Hvorfor nogle fødevareproteiner giver allergi og andre ikke gør, vides endnu ikke, men der er flere bud på hvilke karaktertræk der potentielt kunne bidrage til de allergene egenskaber. Stabilitet over for fordøjelse er en af dem. Denne antagelse er baseret på studier der viser at allergene fødevareproteiner generelt er mere resistente over for nedbrydning end fødevareproteiner der ikke vurderes at være allergene. Dette ledte til konklusionen at stabilitet over for fordøjelse korrelerer med det allergene potentiale. Stabilitet over for pepsinnedbrydning er derfor en et testparameter inkluderet i risikovurderingen af det allergene potentiale af "nye" proteiner i genmodificerede fødevarer. I de senere år er der dog sat spørgsmålstegn ved graden af sammenhæng mellem et proteins stabilitet over for fordøjelse og det allergene potentiale.

Formålet med nærværende ph.d.-afhandling var at undersøge den sensibiliserende evne af fordøjelsesprodukter fra jordnøddeallergenet Ara h 1 samt komælkeallergenet *β*-lactoglobulin (BLG) i en Brown Norway (BN) rottemodel. Endvidere var formålet at sammenligne IgE-bindingsepitoper for intakt og fordøjet Ara h 1.

Dette blev udført ved at nedbryde Ara h 1 og BLG i en *in vitro* model der efterligner den humane gastrointestinale fordøjelsesproces. Den gastriske *in vitro* fordøjelse blev udført med immobiliseret pepsin ved pH 2,5 i 120 minutter, mens den intestinale *in vitro* fordøjelse blev udført med immobiliseret trypsin og chymotrypsin ved pH 6,5 i 15 minutter. Fraktioner af fordøjelsesprodukter blev fremstillet ved en separation of peptidfragmenter via gel filtreringskromatografi (GPC) i henhold til størrelser af peptider samt aggregater heraf. En grunding karakterisering af de intakte allergener samt diverse fordøjelsesprodukter herfra blev udført ved omvendt fase HPLC, MALDI-TOF massespektrometri, aminosyreanalyse samt GPC. For at undersøge den sensibiliserende evne af de intakte allergener, fordøjelsesprodukter og fraktioner herfra, blev grupper at rotter immuniseret ved i.p. injektion. Det specifikke antistofniveau i serum blev bestemt ved ELISA'er, RBL-assay og aviditetsanalyser. Sammenligning af IgE-bindingsepitoper på intakt og fordøjet Ara h 1 blev udført ved "competetive immunoscreening" af et "random phage-displayed peptide library" efterfulgt af en mapning af epitop-efterligningerne på overfladen af Ara h 1 molekylet. Foruden sera fra immuniserede rotter blev sera fra fem jordnøddeallergiske patienter anvendt.

Nedbrudt Ara h 1 fra både den gastriske og den gastrointestinale fordøjelse havde sensibiliserende evne i BN rotterne. Mens peptidfragmenterne af Ara h 1 fra den gastriske proteolyse var op til 4 kDa havde peptidfragmenterne fra den gastrointestinale proteolyse en størrelse på op til 2 kDa. Peptidfragmenterne fra begge fordøjelsesprocesser viste sig dog at være aggregeret til komplekser af større størrelser. Den sensibiliserende evne af fordøjet Ara h 1 forsvandt når peptidfragmenterne blev opdelt i fraktioner. Epitopmapningsstudiet af intakt og fordøjet Ara h 1 resulterede i identificering af konformationelle Ara h 1-specifikke IgE-bindingsepitoper, hvoraf flere overlevede den gastrointestinale fordøjelse. For de jordnøddeallergiske patienter blev der defineret fem motiver som tegnede sig for mere end 65% af alle identificerede epitop-efterligninger og blev fundet for både det intakte og det nedbrudte Ara h 1. Fordøjet BLG som indeholdt peptidfragmenter af størrelser op til 4,5 kDa kunne i modsætning til fordøjet Ara h 1 ikke inducere et allergisk respons i BN rotterne. Derimod tydede sensibiliseringsstudiet med fordøjet BLG på at peptidfragmenterne heri besad tolerogene egenskaber, da de ved en co.-immunisering med det intakte BLG forårsagede et stærkt reduceret respons i forhold til BLG alene.

Resultaterne præsenteret i denne afhandling demonstrerer at selv meget små peptidfragmenter, som oprindelig var anset for at være for små til at besidde allergene egenskaber, kunne agere som "komplette"

allergener og indeholde både evnen til at binde IgE, udløse et degranuleringsrespons samt sensibilisere. Dette demonstrerer at en fuldstændig korrelation mellem stabilitet over for fordøjelse og det allergene potentiale ikke eksisterer. Studierne præsenteret i nærværende ph.d.-afhandling indikerer at enten besidder de små peptidfragmenter fra fordøjet Ara h 1 sensibiliserende kapacitet i sig selv eller også er den sensibiliserende evne et resultat af deres aggregering til komplekser af større størrelser. En sandsynlig signifikant betydning af aggregat-dannelser belyses i epitopmapningsstudiet, hvor overlevelse af konformationelle epitoper kun synes sandsynliggjort ved dannelse af sådanne aggregat-komplekser. Set i lyset af disse resultater samt den manglende sensibiliserende evne af de aggregerede peptider delt i fraktioner, synes en aggregering ikke i sig selv at være nok til at forklare den vedholdende sensibiliserende evne. I stedet peger studierne på at måden hvorpå peptiderne aggregere er af stor betydning og at en hypotese kunne være at peptidfragmenterne i fordøjet Ara h 1 er i en formation der symbolisere det intakte molekyle. Mens nogle allergener bibeholder deres sensibiliserende selv efter nedbrydning til små peptidefragmenter, er dette ikke tilfældet for alle allergener. Komælkeallergenet BLG mistede i modsætning til Ara h 1 dets sensibiliserende evne efter *in vitro* fordøjelse. Dette viser at mens én blanding af peptider kan dirigere immunsystemet i én retning vil en anden blanding af peptider sandsynligvis kunne dirigere immunsystemet i modsatte. Samlet viser nærværende studier at flere karaktertræk er medbestemmende for det allergene potentiale af et fødevareallergen og at dette ikke kun er baseret på nedbrydeligheden af proteinet, men også på strukturen af nedbrydningsprodukterne, sandsynligvis i samspil med andre karaktertræk.

Resultaterne præsenteret i nærværende ph.d.-afhandling bidrager til forståelsen af, hvilke egenskaber der bidrager til et proteins allergene egenskaber og øger vores forståelse af de mekanismer der ligger til grund for at dirigere vores immunsystem mod sensibilisering. Det slås dog samtidig fast at det vil være en stor udfordring, måske umuligt, at identificere de egenskaber ved et protein der bestemmer dets allergene kapacitet.

ABBREVIATIONS

ALA	α-lactalbumin
APC	antigen presenting cell
BAT	basophil activation test
BLG	*β*-lactoglobulin
BN	Brown Norway
BSA	bovine serum albumin
CD	cluster of differentiation
CRD	component-resolved diagnostics
DBPCFC	double-blind placebo-controlled food challenge
DC	dendritic cell
eHF	extensively hydrolysed formula
EAST	enzyme allergosorbant test
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
FAE	follicle associated epithelium
FAO	Food and Agricultural Organisation
FEIA	fluorescence enzyme immunoassay
GALT	gut-associated lymphoid tissue
GI	gastrointestinal
HEL	hen's egg lysozyme C
HR	histamine release
IEC	intestinal epithelial cell
Ig	immunoglobulin
IgE	immunoglobulin E
IL	interleukin
ILF	isolated lymphoid follicle
i.p.	intraperitoneal
i.v.	intravenous
M cell	microfold cell
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MW	molecular weight
nd	not described
ns-LTP	nonspecific-lipid transfer protein
OAS	oral allergy syndrome
OT	ovotransferrin
OVA	ovalbumin
OVM	ovomucoid
PC	phosphatidylcholine
pHF	partially hydrolysed formula
PP	Peyer's patch
PPV	positive predictive value
PR-10	pathogenesis-related protein-10
RAST	radioallergosorbent test
RBL	rat basophilic leukaemia
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophorese
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
SPT	skin prick test
STI	soybean trypsin inhibitor
TCR	T cell receptor
TGF	transforming growth factor
Th3 cell	T helper 3 cell
Tr1 cell	T regulatory 1 cell
WHO	World Health Organisation

CONTENT

GENERAL INTRODUCTION

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Food allergy is defined as an immune mediated adverse reaction observed upon ingestion of an otherwise harmless food [1-3]. Food allergy is a major health problem in the Western countries, where it affects around 5-8% of young children and 2-4% of adults [4;5], and appears to be an increasing problem [6-9]. Food allergy is most often due to an immunoglobulin E (IgE) mediated mechanism (type I food allergy) [5;10], where the pathogenesis is composed of two phases. The first phase involves a primary contact with the dietary protein where oral tolerance induction fails or is abrogated, leading to sensitisation, where naïve B cells are primed to become IgE secreting plasma cells. Subsequently, allergen-specific IgE antibodies bind to the high affinity receptor FcεR1 on tissue mast cells and blood basophils. The second phase is a result of reexposure to the offending dietary protein or a cross-reacting protein, which binds and cross-links FcεR1-bound IgE. This leads to a degranulation response of the mast cells or basophils, releasing preformed and newly synthesised mediators, responsible for the symptoms of the allergic disease [11-13].

Among the large number of proteins, that humans eat, only minute proportions are allergens. This may suggest that certain dietary proteins possess specific intrinsic features of allergenicity. Yet there is no absolute answer to the question 'what makes a dietary protein a food allergen?' [14]. Even though the mechanism by which dietary proteins sensitise an individual remains basically unresolved, many food allergens are thought to sensitise through the gastrointestinal (GI) tract. Thus, resistance to proteolysis in the GI tract has received much attention in recent years and is generally thought to be a prerequisite for a protein to sensitise through the mucosal immune system of the GI tract [15-18]. The first systematically evaluation of proteolytic stability of allergenic as well as non-allergenic dietary proteins by means of an *in vitro* digestibility assay was conducted in 1996 by Astwood *et al*. [18]. This study in general showed allergenic dietary proteins to be resistant to peptic digestion in contrast to the non-allergenic dietary proteins which showed to be rapidly digested, leading to the conclusion that a correlation between resistance to digestion and allergenicity exist. This has contributed to the inclusion of pepsin resistance as a test parameter in the decision tree or weight-of-evidence approach used in the safety assessment of novel proteins in genetically modified foods [19-21].

Since the study by Astwood *et al*. [18] major efforts have been used for studying the digestibility of known food allergens and have thrown some doubt about an absolute association between stability to digestion and allergenic potential. Besides an assessment of the proteolytic susceptibility of food allergens, several studies have in addition evaluated the residual allergenicity of the emerging digestion product. By use of immunological assays, studies have assessed the IgE binding capacity and/or the eliciting capacity of the generated peptide fragments. However the sensitising capacity of degraded food allergens is not well investigated, probably as a result of the ethical impossibility for studying such in humans.

Gastrointestinal tract – the fate of dietary proteins

Every day humans eat numerous of different food proteins. Understanding the fate of such dietary proteins from the moment they reach the mouth until they are taken up and presented to immune cells in the gut-associated lymphoid tissue (GALT) is essential in food allergy, since many food allergens are believed to sensitise via the GI tract and because of the role digestion may play in determining the allergenic potential of such dietary proteins [15;22].

The GI tract is the largest organ in the body conducting several functions in digestion and absorption of nutrients as well as maintaining the immune homeostasis in discriminating between adverse foreign matter and harmless food components, microorganisms and self-antigens [23]. However, occasionally humans are sensitised to harmless food proteins, developing food allergy. The immunological mechanisms involved in the sensitisation of individuals towards dietary proteins remain poorly understood but it is thought that allergens or digestive products hereof must cross the intestinal mucosa in order to interact with the immune system of the gut, the same prerequisite thought for an allergen to elicit an allergic reaction in individuals already sensitised [22;24].

Digestive system

Ingested food undergoes complex series of digestive processes in order to extract the nutrients essential for maintenance of the health. Proteins are broken down by hydrolytic enzymes originating in the stomach, pancreas, and small intestine [25]. In the stomach proteins are exposed to proteolysis by different pepsins [25;26]. Pepsins have a wide specificity, preferentially cleaving peptide bonds between hydrophobic and aromatic amino acid residues like, phenylalanine, tyrosine, and tryptophan [27;28], with an activity optimum around pH 3.5 [29]. The gastric pH is thought to be between 1.2 and 3.0 [30-32] but may vary further during the ingestion of a meal, because of influence by the volume and meal content [22;32]. The period of time the food stays in the stomach also varies. The average transit time is estimated to around 1 to 2 hours [33]. Subsequently, the gastric digests are released into the small intestine, where the pH is around 6 to 6.5 [34]. Here the gastric digests are subjected to proteases and peptidases, produced by the pancreas, such as trypsin and chymotrypsin, or produced by the brush border of the intestinal mucosa [25;30]. While trypsin cleaves peptide bonds at the carboxyl side of the basic amino acids arginine and lysine, chymotrypsin cleaves peptide bonds where the carbonyl group is aromatic, like in the amino acids phenylalanine, tyrosine and tryptophan [25;35], with an activity optimum at pH 8 and 7.8, respectively [36-38]. In the stomach and small intestine digests are mixed with surfactant such as phosphatidylcholine (PC) and bile salts, which may influence the digestibility of proteins [39-43]. The susceptibility of dietary proteins to digestion varies greatly but the goal is to achieve a mixture of amino acids and small peptides that can rapidly and efficiently be absorbed over the intestinal mucosa and serve as nutrients for the body [25]. While the majority of proteins are digested to amino acids and small peptides, some larger immunologically active fragments may survive the digestion process, and be absorbed and presented to the immune system of the Peyer's patches (PPs). The approximately transit time down the duodenum to the site of the first PPs is 15 min [41]. Also small quantities of intact protein may escape the digestion process, which has been shown for the cow's milk allergens *β*-lactoglobulin (BLG) [44-46] and bovine serum albumin (BSA) [47] and the hen's egg allergen ovalbumin (OVA) [45;46;48;49].

Several *in vivo* studies of human digestion have been performed [50;50-52]. However, such studies are technically and ethically difficult to conduct and at the same time expensive [22]. Therefore, several attempts have been made to develop *in vitro* models simulating the human digestion process [39;53-55]. In addition, *in vitro* digestion models have been made which did not attempt to mimics the human digestion process [18;56]. Little consistency appears to exist for the digestion conditions, as great differences in the model systems are evident on e.g. pH, enzyme to protein ratio, digestion time, or addition of surfactant. However it is important to notice that no single perfect *in vitro* digestion model can be made, because the extent of digestion significantly varies from person to person and are influenced by factors such as age, health and medication status [30;57].

Absorption

Most dietary proteins are digested to amino acids and small peptides which are absorbed through the intestinal mucosa by electrogenic and sodium-dependent transporters [58]. Larger immunologically active peptides and intact proteins may also be absorbed across the intestinal epithelial barrier to reach the specialised inductive sites of the mucosal immune system. Only a single layer of intestinal epithelial cells (IECs) separates the intestinal lumen from the GALT, yet, several routes are suggested for antigen transport (Figure 1) [59-61]. Proteinous antigens may cross the epithelial barrier by transcytosis through enterocytes where three distinct routes exist. Most proteins or peptides are sorted into lysosomal compartments for (further) degradation before release [62;63] but may also be transported through a separate non-degradative pathway [63;64]. Additionally, in food sensitised individuals enhanced transepithelial allergen transport mediated by specific IgE bound to the low affinity receptor FcεRII (CD23) has been suggested [65;66], protecting the allergens from lysosomal breakdown [67]. Antigens may also be taken up by specialised microfold (M) cells which are restricted to the follicle associated epithelium (FAE) covering the immune inductive sites of PPs and isolated lymphoid follicles (ILFs) [60;68;69]. M cells are characterised by reduced activity of intracellular lysosomes and an intra-pocket structure at basal site where antigen presenting cells (APCs) and lymphocytes are located, allowing M cells to easily take up antigens from lumen and transport them without digestion and processing [63;69]. Furthermore, dendritic cells (DCs) penetrate the epithelial layer to sample antigens in the intestinal lumen and migrate to local or distant lymphoid tissue [70]. There are also situations where small proteinous antigens may enter the inductive site of the mucosal immune system by paracellular diffusion through the intestinal epithelial barrier, where the IEC are joined by tight junctions. This pathway seems to be negligible under physiological conditions [63].

The route of antigen entry is not solved, due to the complexity of the GALT and the difficulty in studying a process with a high number of variables [23]. The location of antigen uptake may thus depend on different factors such as size of antigen, maturity of mucosa, medical status and microflora [63;71]. However, the site, pathway and mechanisms by which the dietary proteins or digestive products hereof enter the inductive site of the mucosal immune system may be of great importance for the immune response to that dietary antigen [24;58;61;63;72;73]. Although the normal immune response to dietary proteins is oral tolerance, proteins or digestive products are sometimes responsible for an adverse stimulation of the mucosal immune system, resulting in sensitisation [63].

Inductive system of the GALT

In addition to the mode of antigen uptake in the GI tract, the way in which the antigen is presented to the effector immune cells in the GALT, also plays an important role in the development of oral tolerance versus sensitisation to dietary antigens.

The GALT is divided into organised lymphoid tissue, which is the inductive site and consists of PP, ILF and mesenteric lymph node (MLN), and the non-organised lymphoid tissue, which is the effector site and consists of lymphocytes scattered throughout the epithelium and lamina propria (Figure 1) [60;61;72]. PPs are specialised lymphoid follicles, consisting of B cell follicles surrounded by T cell areas which are infiltrated by DCs [60]. PPs are thought to have an essential role in the induction of either antigen-specific immune responses or oral tolerance [74;75].

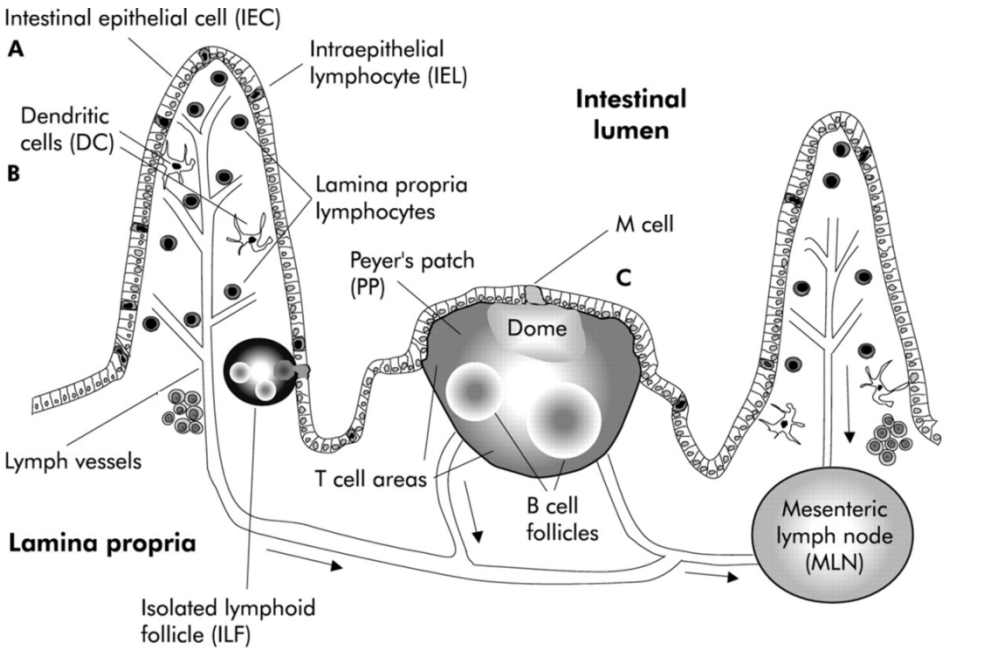

Figure 1. Overview of the mucosal immune system of the intestine. GALT can be divided into induction sites, which consist of Peyer's patches (PP), isolated lymphoid follicles (ILF) and mesenteric lymph nodes (MLN) and effector sites, which consist of lymphocytes scattered throughout the epithelium and lamina propria. Dietary antigens may access the mucosal immune system by means of three different cell types: **A**. Antigens can cross the intestinal epithelial cells (IEC) through different transcellular routes, **B**. Antigens can be sampled by dendritic cells (DC) that extend processes through the epithelium and into the lumen, or **C**. Antigens can be taken up by microfold (M) cells overlying PPs and ILFs [60;61;72]. Modified from Spahn and Kucharzik [60].

After antigen transport across the epithelial barrier, proteinous antigens are readily taken up by the underlying APCs such as DCs located in the organised lymphoid tissue of e.g. PP. APCs process and present antigenic peptides in association with major histocompatibility complex (MHC) class II molecules for recognition by specific T cell receptors (TCRs) expressed on the surface of naïve T cells. Priming of T cells occurs either locally or after migration of APCs to distant sites of the immune system [72;76]. APCs have a fundamental role in determination of whether an immune response is directed towards food allergy or oral tolerance depending on the presence of appropriate signaling by co-stimulatory surface molecules and by cytokine secretion by the APC [23;61].

Food antigens taken up by IEC may be processed and presented on MHC class II molecules on their surface, indicating that IECs might function as APCs [77;78]. Several studies have shown that this presentation by IECs may contribute to the development of oral tolerance, since IECs do not express the co-stimulatory molecules required for full T cell activation [72;78;79]

Oral tolerance is generally induced to food proteins and is defined as a state of unresponsiveness to a specific antigen, after prior exposure to that antigen by the oral route [23;58;61]. Multiple mechanisms may be involved in the induction of oral tolerance in the mucosal immune system of the GI tract, including (1) T cell anergy, mediated through TCR ligation in the absence of appropriate co-stimulatory signals provided by ligation of receptors on T cells (CD28) with receptors on APCs (CD80 and CD86) and by soluble cytokines secreted by APCs [80;81]. This results in abolishing of T cell capacity for production of their own growth factor interleukin (IL)-2 upon restimulation [82]; (2) T cell deletion, which occurs by means of apoptosis [83]; and (3) T regulatory cells, inhibiting immune responses through secreted or cell surface-

bound suppressive cytokines [61]. T regulatory cells can be divided into different subgroups: suppressor CD8+ cells, T helper 3 (Th3) cells, T regulatory 1 (Tr1) cells, and CD4+CD25+ cells [76;84]. Mechanisms for suppression may be different for the different regulatory T cells, where CD8+ cells and Th3 cells suppress mainly through secreted transforming growth factor (TGF)-β [85;86], Tr1 cells suppress mainly through secreted IL-10 [87], and CD4+CD25+ suppress possible through surface-bound TGF-β [88]. Regulatory T cells may work locally or migrate to distant lymphoid organs, where they inhibit the generation of effector cells [84]. By producing immunomodulating cytokines, regulatory T cells are also important for keeping the Th1/Th2 profile balanced.
Studies in experimental animal models have shown that oral tolerance can be induced after administration of either a single high dose of antigen or repeated lower doses [89;90]. Different mechanisms are involved in the two types of tolerance, termed high-dose tolerance and low-dose tolerance, respectively (Figure 2) [61]. Whereas high-dose tolerance is mediated by lymphocyte anergy [89] or deletion [83], low-dose tolerance is mediated by regulatory T cells [83;89]. However, low- and high-dose tolerance may not necessarily be reciprocal and likely have overlapping functions [58;76;84].

Abrogation of oral tolerance or failure to induce oral tolerance may result in development of adverse immunological reactions to dietary proteins [61;76;91]. The immune system will be primed, leading to cell-mediated immunities or production of antibodies reacting with the ingested dietary antigen on subsequent exposures.
When naïve Th cells are primed by APCs providing appropriate signals through surface expressed co-stimulatory molecules and secreted cytokines, they differentiate into either Th1 or Th2 cells. Optimal Th cell activation requires, besides TCR ligation with MHC/peptide complex, co-stimulatory signaling through the interaction of CD28 on the Th cell and CD80/86 on the APC (Figure 2) [76;82;92]. The direction of Th cell differentiation to either Th1 or Th2 cells depends on the specificity of the signals provided by the APC where IL-4 seems to have the pivotal role in the differentiation to Th2 cells [93;94]. Th2 cells are defined on basis of their restricted cytokine profiles [95], which includes the production of the cytokines IL-4, IL-5 and IL-13 [96].

When membrane-bound immunoglobulin (Ig) of naïve B cells come in contact with specific dietary antigens, activation of the surface molecule CD40 by ligation with the Th2 molecule CD40L and IL-4 and IL-13 secreted by activated Th2 cells promote germ line ε transcript for Ig isotype class switching in B cells. Naïve B cells differentiate and proliferate into active plasma cells synthesising and secreting antigen-specific IgE [97;98]. Lymphocytes activated in the GALT leave through the draining lymphatics and reach the MLN, where they stay for a period for further differentiation, before migration into the bloodstream [72].

To this day the immunological mechanisms involved in food allergy versus tolerance induction remain poorly understood but is realised to depend on a complex network of communicating immune cells, which are the focus of intensive research. New knowledge in the mechanisms underlying food allergic sensitisation would be helpful for the development of new prophylactic and therapeutic strategies for food allergy [76].

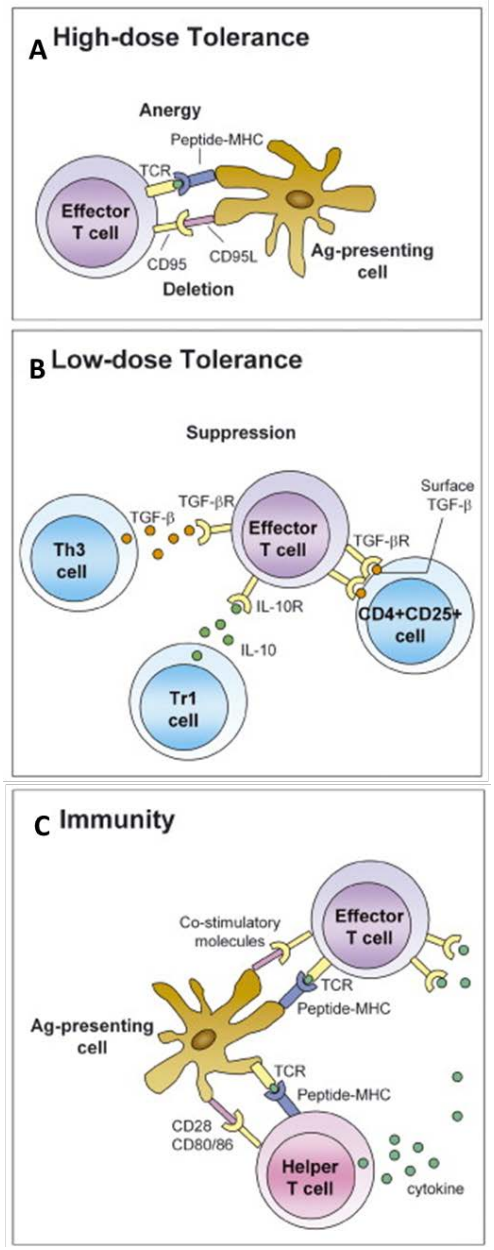

Figure 2. Mechanisms of oral tolerance versus immune response. When an antigen encounters the immune system of the gastro-intestinal (GI) tract, different mechanisms may be activated. Several factors may influence the outcome of the response to a dietary antigen. Some are related to the antigen, including the dose and nature of the antigen, while other factors are related to the host, including age, genetics and intestinal flora content. In normal circumstances oral tolerance to dietary proteins is induced. **A**. High-dose of antigen may lead to T cell receptor (TCR) ligation with peptide/major histo-compatibility complex (MHC) in the absence of co-stimulatory molecules or in the presence of inhibitory molecules (CD95 and CD95 ligation), where anergy or deletion will be the result. **B**. Low-doses of antigen cause activation of regulatory T cells, which may prevent immune responses either through secretion of or by cell-bound suppressive cytokines (interleukin (IL)-10 and transforming growth factor (TGF)-β). **C**. Initiation of an immune response requires the binding of TCR to peptide/MHC complexes in the presence of adequate co-stimulatory molecules (CD80 and CD86) as well as cytokines [61;91]. Modified from Chehade and Meyer [61].

Food allergy – an adverse response to dietary proteins

Adverse reactions to foods can be defined as any anomalous reaction resulting from ingestion of a food or a food ingredient. Adverse reactions to foods can be divided into toxic and non-toxic reactions (Figure 3). While toxic reactions may occur in any individual ingesting an appropriate amount of the toxic compound, non-toxic reactions only occur in susceptible individuals [1] and is often referred to as 'food hypersensitivity' [2]. Non-toxic food reactions can be divided into immune mediated reactions also termed 'food allergy' [1;2] and non-immune mediated reactions also referred to as 'food intolerance' [1] or 'non-allergic food hypersensitivity' [2]. Non-immune mediated reactions are adverse physiologic reactions caused by some distinctive characteristics of the individual, such as metabolic disorders [3;10;76], and can

be divided into enzymatic, pharmacologic and undefined reactions [1]. Immune mediated reactions can be further divided into IgE mediated reactions also referred to as 'Type I food allergy' and non-IgE mediated reactions [1]. While IgE mediated food allergy are responsible for most food allergic reactions and is characterised by the presence of food-specific serum IgE antibodies [5;10;76], non-IgE mediated food reactions are associated with cell-mediated mechanisms or antigen-specific antibodies other than IgE (Figure 3) [2].

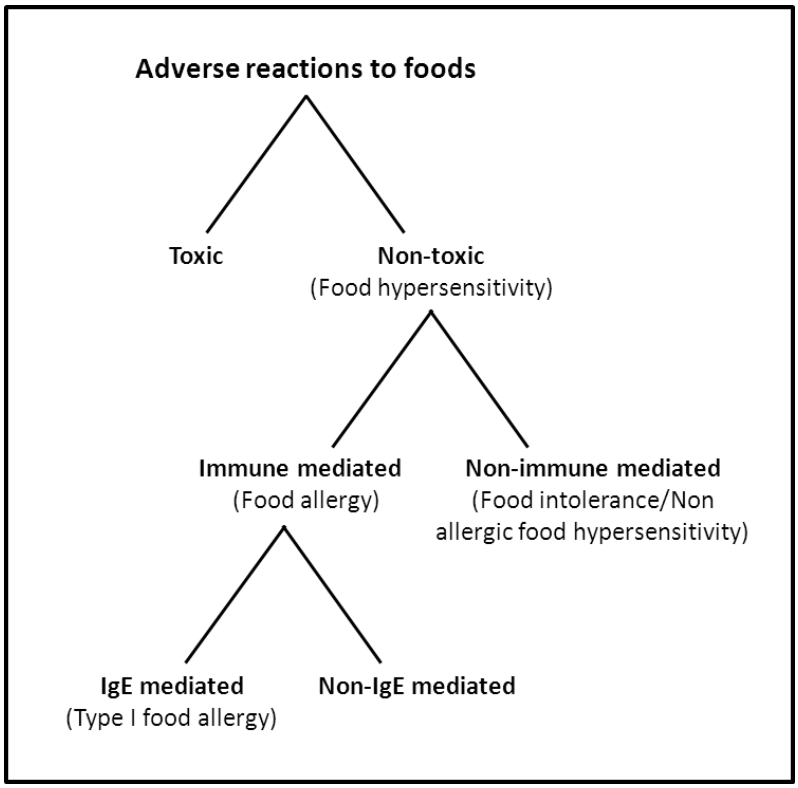

Figure 3. Overview of adverse reactions to foods

The factual prevalence of IgE mediated food allergy is unknown because appropriately performed worldwide epidemiologic studies have not been conducted [1]. There are global variations in food allergy prevalence but it is suggested to affect around 5-8% of young children and 2-4% of the adults in the Western countries [4;5], where it also gives the impression of being an increasing problem [6-9] or achieving increasing attention [8]. Various foods are responsible for allergic responses, however, only a few accounts for the majority of the reactions, including milk, peanut, egg, tree nuts, shellfish, fish, wheat and soy [4;5]. Additionally, the number of identified incriminating foods continue to increase, which could either be the result of the globalisation and thereby the introduction of new foods containing potential new allergenic proteins [99] or simply an increased attention for identification of dietary proteins causing the allergic responses. The increased attention that food allergy has achieved in recent decades as well as the introduction of genetically modified food products into the market have led to the recommendation that novel food products should be subjected to a careful and complete safety assessment including the evaluation of potentially allergenicity, before commercialisation [16]. The Food and Agricultural Organisation (FAO)/World Health Organisation (WHO) established in 2001 a decision-tree approach for predicting the potential allergenicity of novel proteins in genetically modified foods [19]. Later the Codex Alimentarius [21] and the European Food Safety Authority (EFSA) [20] recommended the use of a weight-of-evidence approach.

Several factors influence the development of IgE mediated allergy, including genetics, environmental factors and antigen related features [61;100;101]. An important determinant is genetic predisposition, however, no specific genetic markers have been identified, suggesting that multiple genes have implication for development of food allergy, each probably playing only a tiny role [2]. Another possibility is that epigenetics play a significant role [102;103]. The fact that the increase in prevalence of allergic diseases in Western societies, which cannot solely be accounted for by the genetics, concur with a reduction of infections in early childhood, has led to a theory of a causative relationship [104;105]. Such theory is collectively known as the hygiene hypothesis and were formulated first time in 1989 [106]. However, the implication that increased environmental sanitation is associated with higher incidence of allergy, remain to be fully documented [100]. Other factors contributing to the development of allergy are suggested to be the age at which solid food is introduced, breast versus formula feeding, intestinal microflora composition, degree of gastrointestinal infection, intestinal permeability, mechanisms and site of intestinal antigen absorption, and adjuvant effects from e.g. tobacco smoke [61;100;101].

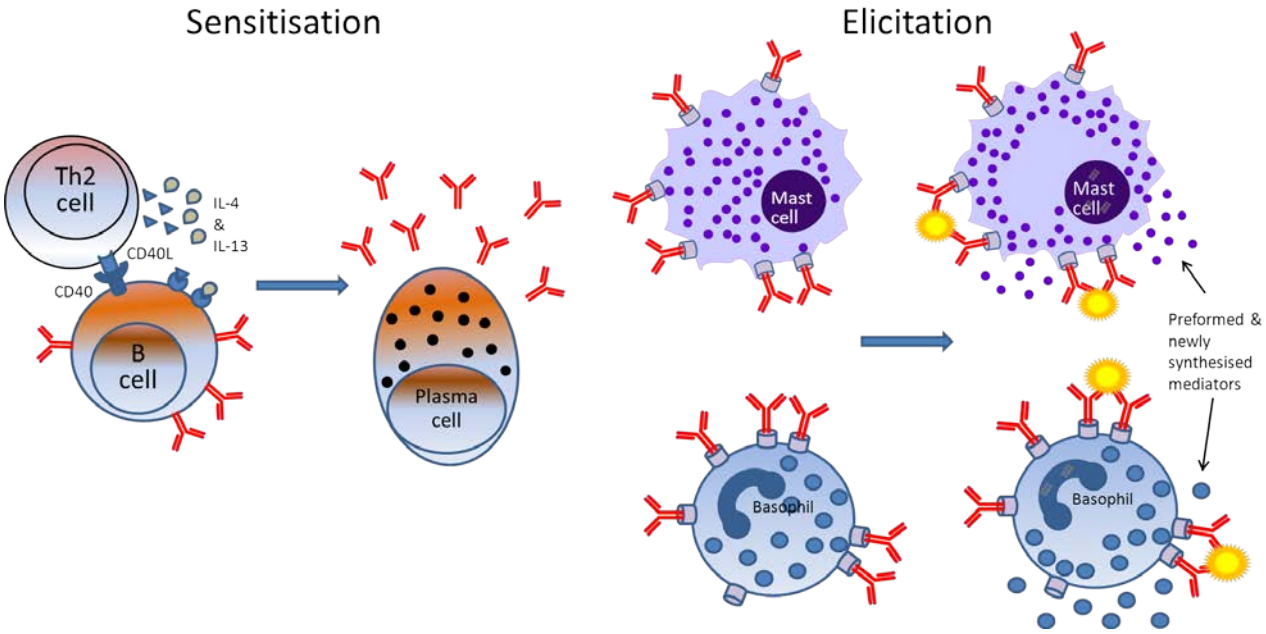

Figure 4. Overview of mechanisms leading to allergic reactions. The pathogenesis of IgE mediated food allergy has two phases; a sensitisation and an elicitation phase. Sensitisation usually occurs by the primary contact with a given allergen, where naïve B cells are primed to become IgE secreting plasma cells. Differentiation and proliferation to plasma cells require besides allergen recognition of membrane-bound IgE also signals provided by Th2 cells in form of ligation of CD40 by CD40L and cytokine secretion. Elicitation occurs upon reexposure to the same or a cross-reacting allergen, which cross-links FcεRI-bound IgE on mast cells and basophils. This activation leads to release of preformed and newly synthesised mediators inducing the allergic symptoms characteristic of food allergy.

The pathogenesis of IgE mediated food allergy is composed of two phases; (1) A primary contact with the dietary protein where oral tolerance induction fail or is abrogated, leading to sensitisation, where naïve B cells are primed to become IgE secreting plasma cells. Food-specific IgE antibodies then bind to FcεRI, the high affinity receptor, expressed on tissue mast cells and blood basophils, and (2) upon subsequent contact with the incriminating protein, binding and cross linking of FcεRI-bound IgE occur. This leads to

degranulation of mast cells or basophils, releasing preformed and newly synthesised mediators, such as histamine, *β*-hexoaminidase, cytokines and proteases (Figure 4) [11-13;76].
Release of mediators is responsible for a variety of symptoms, ranging from mild local reactions to severe systemic anaphylactic reactions, which could potentially be life-threatening [4;10]. These allergic reactions occur within minutes to hours after ingestion of the incriminating food [107;108]. Besides the physiological consequences, food allergy may also have significant effect on the psychological wellbeing of the food allergic patient.
Several diagnostic techniques are available for the diagnosis of IgE mediated food allergy. Double-blind placebo-controlled food challenge (DBPCFC) is the gold standard but is often replaced by or performed in combination with open or single-blind food challenges, skin prick test (SPT), *in vitro* assays for determination of specific IgE antibodies, case history and/or elimination and re-introduction of diet [1;4]. Diagnosis may be complicated by the fact that symptoms representative of IgE mediated food allergy may appear in patients without detectable levels of specific IgE [109;110] as well as detection of specific IgE does not necessarily correlate with clinical symptoms [108;111;112]. Novel diagnostic methods, focusing on protein and epitope specificity are under investigation [4].
No cure for food allergy exists. Currently, there are no therapeutic approaches of documented value [113;114] and avoidance of the offending foods is the only reliable management of food allergy [4;5]. However, a number of novel therapeutic strategies targeting food allergy are under investigation, including both food allergen specific and non-specific strategies [1;114]. The approaches undergoing the most extensive research are oral and sublingual immunotherapy, where doses of the dietary protein are given in progressively increasing quantities toward a steady dose, for induction of desensitisation. Studies to date indicate that immune therapy may indeed induce desensitisation but it remains unclear whether tolerance is achieved. Other food allergen-specific strategies include, epicutaneous immune therapy, peptide immune therapy, plasmid DNA immune therapy. Allergen non-specific strategies include probiotics and prebiotics, anti-IgE antibodies, Chinese herbal medicine, anti-cytokines and toll-like receptor agonists [113;114].

Food allergens

An allergen is defined as the antigenic molecule giving rise to an allergic response [1] and is virtually always proteinaceous in nature [15]. A food allergen possess three distinct molecular properties; (1) the property to bind IgE antibodies, (2) the property to elicit an allergic reaction, and (3) the property to sensitise an individual [115]. Aalberse [115] states that for an allergen to be complete it must possess all three distinct properties. Not all allergens are complete allergens. Well-known examples of such incomplete allergens are the dietary proteins homologous to the birch pollen allergen Bet v 1, such as Mal d 1 from apple, Pru av 1 from cherry, Cor a 1 from hazelnut and Api g 1 from celery, which are known to elicit allergic reactions but do not usually sensitise [115-117].
Allergens can be defined as being either major or minor. A major allergen is defined as one, recognised by IgE from more than 50% of individuals sensitive to the particular food from where the allergen origin [118]. Aalberse [115] however claims that this definition is unsatisfactory, since e.g. a major allergen is not necessarily synonymous with a major risk.

More than 700 different allergens are defined, and of these it appears from the Informall Database on Food Allergies (http://foodallergens.ifr.ac.uk/allergenlist.html) and IUIS Allergen Nomenclature (http://www.allergen.org) that more than 200 are derived from foods. The food allergens originate from approximately 90 different species, with around 70% being of plant origin and around 30% being from

animals. Although humans in general eat a very varied diet, only few foods account for nearly half of the identified allergens, which are milk, peanut, egg, tree nuts, shellfish, fish, wheat and soy. Hypothetically, all food containing proteins could induce an allergic reaction, although foods appear to vary greatly in their likelihood of inducing allergy.

Among the large number of proteins, eaten by humans, only minute proportions are allergens, suggesting that allergens hold special features of allergenicity. No definitive answer to the question 'what makes a dietary protein a food allergen?' exists, though it is clear that some dietary proteins are intrinsically more allergenic than others [14]. In general food allergens have been suggested to be water-soluble glycoproteins with a molecular weight (MW) of 10 to 70 kDa, that are abundant in the food and are stable to treatment with heat, acid and proteases [14;17;119]. However, many food allergens do not share such characteristics [17;119-121] while proteins considered not to cause allergy can be identified with those characteristics [119;121]. It appears likely that many factors may contribute to the overall allergenicity of any given protein, none of which is unique, but that some characteristics are more common among proven allergens than among other proteins considered to be non-allergenic, as stated by Huby *et al.* [14]. The only definitive requirement for a food protein to be an allergen is to possess IgE binding epitopes.

Since no single characteristic of a dietary protein is sufficient for predicting its allergenic potential, it is recommend that the risk assessment process of novel proteins in genetically modified foods should implement a stepwise case-by-case approach that takes into account several types of information [16], including; (1) evaluation of the source of the gene, (2) the sequence homology of the newly introduced protein to known allergens, (3) the expression level of the novel protein in the modified food, (4) serum screening for the reactivity with IgE from the serum of individuals with allergy to the source of genetic material or (5) from the serum of individuals with allergy to materials that are related to the source material for the gene, (6) test for resistance to pepsin degradation [19-21] and (7) cell based assays [20]. In addition, animal models have been suggested for prediction of the sensitising capacity of the novel proteins [19].

IgE – the main player in food allergy

Immunoglobulins (Igs), also designated antibodies, are produced by plasma cells and maintain the key function of the humoral immune system. Antibodies are grouped into five isotypes, according to the heavy chain they possess, where each isotype is developed to perform distinctive roles. Antibodies hold a hypervariable region, in which the paratope is localised, the site of the antibody responsible for interaction with an antigenic molecule, allowing for generation of millions of different antibodies. Each antibody clone possess a unique binding capability. Consequently this huge variety of antibodies allows the immune system to recognise an equally immense diversity of antigenic molecules, with the main function of clearing the antigens from the human body.

IgE antibodies were discovered in 1966 [122] and found to play the essential role in type I allergy [122;123]. IgE performs the immune response by binding to the high affinity FcεRI expressed on mast cells and basophils. Cross-linking of the receptor-bound IgE antibodies causes degranulation and release of mediators responsible for the symptoms of the allergic reaction [11;13]. IgE is the least abundant antibody isotype in serum, with a concentration of 150 ng/mL [11] in comparison to e.g. 10 mg/mL for IgG [11;124]. Free IgE has a short half-live of only a few days [11;12;125]. The half-live may be increased to about two weeks when bound to the FcεRI on mast cells [13;125]. Both the concentration of IgE as well as the half-live may be increased in atopic individuals, persons predisposed for developing type I allergy [125]. The

possibility exists for an IgE mediated response to last for years without any allergen stimulation, probably as a result of long-lived IgE producing plasma cells [98].

Food allergy diagnosis often involves detection of specific IgE antibodies in combination with a recording of the patient's clinical history and physical examination. The presence of specific IgE antibodies can be assessed by a series of different *in vivo* and *in vitro* testing methods [108;126]. Food challenges and SPT are commonly used *in vivo* methods performed for assessment of food-specific IgE [108;126;127]. IgE antibodies of particular food specificity can be measured in sera or plasma from individuals sensitised to that food using various *in vitro* testing methods, such as radioallergosorbent test (RAST), enzyme-linked immunosorbent assay (ELISA), fluorescence enzyme immunoassay (FEIA), enzyme allergosorbent test (EAST), immunoblotting or the commercially available ImmunoCAP [126]. Allergen-specific IgE can be detected by component-resolved diagnostics (CRD), which allows for detection of IgE, to specific allergens and may be a future commercially available useful tool [127]. Other *in vitro* tests used in the diagnosis of food allergy are cells based assays, such as basophil activation test (BAT), basophil histamine release (HR) test or humanised rat basophilic leukaemia (RBL) cell assay. Besides detection of specific IgE antibodies, these tests provide an evaluation of the biological functionality of the specific IgE [126].

Symptoms representative of type I food allergy may appear in patients without elevated levels of specific IgE. In addition presence of elevated levels of specific IgE may be observed in subjects without a clinical active allergy and only points to the occurrence of sensitisation. Presently, only food challenge tests with the offending food, provide reliable prediction of a clinical active type I food allergy in patients, while no *in vitro* tests at the moment seems to sufficiently predict the clinical reactivity although the outcome of the *in vitro* test may be representative of the associated risk [108].

There is still a lack of knowledge about causal relation between antibody characteristics and the food allergic phenotype as well as epitope characteristics and the food allergic phenotype.

Epitopes

The word epitope was first defined by Jerne [128] in 1960 as an antigenic determinant or region of a molecule recognised by an antibody. At the present time epitopes describe the binding site of a molecule at the amino acid level for both B and T cells. Epitopes can be categorised as either linear or conformational based on the vicinity of the involved amino acids in the primary structure constituting the epitope [129-132]. The first is the simplest epitope as the amino acids comprising the epitope are resulting from a contiguous stretch of the primary sequence. Most T cell epitopes appear to be in this category [133-135]. On the other hand, most B cell epitopes are thought to be conformational, involving amino acids from two or more stretches that are distant from one another in the primary sequence but brought together by structural folding of the polypeptide backbone [24;115;136-138].

Definition of epitopes appears unclear. As Van Regenmortel [131;138] and Arnon and Van Regenmortel [132] outline, there is no clear boundary at the amino acid level for those residues that comprise an epitope. An epitope is identified by its ability to bind antibodies but there is no evidence that each amino acid in the binding area necessarily interacts with the antibody [131;132]. So even though epitopes are suggested to consist of at least 8 amino acid residues, energy calculations have indicated that as few as 5-6 amino acids are the actual contributors to the binding between epitope and antibody molecule [133;138;139]. It has been estimated that by immunisation of an individual by a protein-antigen, more than 100 different antibody clones can be formed. They may differ in their epitope specificity, though be directed against the same epitope areas, so that the entire molecule could be covered by many overlapping epitopes [140;141]. In addition, since only a limited degree of similarity between two structures is sufficient to allow the same antibody to bind, 'true' cross-reactivity may occur where a given antibody reacts with

another epitope than the one for which the antibody was originally developed. The ability of an antibody to react with more than one epitope resembles the inherent multi-specificity of an antibody [131]. Upon reexposure to the same antigen affinity maturation occurs, where progressively higher affinity B cell clones are generated, as a result of somatic hypermutation and antigen-specific selection of high affinity B cells [142-145].

Different methods for identification of specific B cell epitopes exist. For experimental reasons, the number of identified linear epitopes by far exceeds the number of identified conformational epitopes. Most of the mapping techniques are based on analysis of IgE binding to peptides derived from the primary sequence of the allergen [136;146], thereby only allowing for identification of linear epitopes. Identification of conformational epitopes requires more elaborated methods, such as X-ray crystallography, site-directed mutagenesis or phage display technology [136;138;146]. However, the only complete method for defining an epitope involves measurement of the crystal structure of an allergen in complex with an antibody [133].

Great diversity in the patterns of IgE epitope recognition have been seen in allergic patients, which may be elucidated by the individual development of antibody specificities, the polyclonal origin and the individual progression of affinity maturations.

IgE epitope mapping of food allergens may provide valuable information regarding patient's clinical history and contribute to food allergy diagnosis and treatment and as a tool for detecting candidate biomarker for persistency, severity or tolerance induction [147;148]. Also IgE epitope identification may be of relevance for design of allergy peptide vaccines [149], for design of recombinants for immunotherapy [146;150] and for the general understanding of what makes a protein a food allergen, an important issue for predicting the allergenic potential of novel proteins in genetically modified foods.

Antibody characteristics in relation to the allergic phenotype

At present no identified antibody characteristics and no identified structural features of IgE binding epitopes seem to be associated with the phenotype of the food allergic disease.

As specific IgE is the main player in food allergic reactions, research has been made to find correlations to the allergic phenotype. Whereas the presence of specific IgE in serum does not associate with an active clinical food allergic disease, studies have shown that an increasingly higher concentration of specific IgE correlates with an increasingly greater likelihood of clinical reactions upon ingestion of the given food to which the IgE is specific [151-153]. A measure of the specific IgE level that could predict clinical reactivity would be desirable as an alternative to DBPCFC. The predictive value of specific IgE has been examined in numerous studies [151;153-155]. These studies indicate that calculation of a predictive decision points for instance for a 95% positive predictive value (PPV) of specific IgE should be established for each food separately. In general PPVs for specific IgE have been suggested to be a useful parameter for diagnosing symptomatic allergy [154;155]. However, the PPV of the specific IgE may come out very differently for the same foods in separate studies, where for example the 95% PPV for hen's egg-specific IgE ranged from 1.35 to 59.2 kU_A/L [151;153-155]. High levels of specific IgE have been reported to relate to a persistent food allergic phenotype compared to a lower level of specific IgE in patients shown to outgrow their food allergy [155-159]. For example, Savilathi *et al*. [156;157] showed that patients with persistent cow's milk allergy had higher cow's milk specific IgE at the time of diagnosis than patients who later became tolerant. On the other hand conflicting results exist for the correlation between levels of specific IgE and severity. Lewis *et al*. [160] and Hourihane *et al*. [161] reported a significant correlation between levels of specific IgE and severity of the food allergic disease, while Sampson *et al*. [151] reported that such correlation do not exist.

The influence of the total IgE level has also been investigated, and it generally seems that an elevation of total IgE is positively correlated with elevated specific IgE. Elevated levels of total IgE does not necessarily associate with an clinical active allergic phenotype [152], but it generally appears that a high level of total IgE correlate with an increased risk of having a persistent food allergic phenotype [155;162]. Montesinos *et al*. [155] for example showed that the initial mean level of total IgE of individual with persistent hen's egg allergy was more than twice as high as the mean total IgE level of individuals with transient hen's egg allergy [155]. However, other studies showed that such correlation is far from general [163]. Also the ratio of specific IgE/total IgE has been examined, and was shown to correlate with the outcome of oral challenges, so that higher ratios correlated with an increased likelihood of symptomatic food allergy [164;165]. Yet, the overall conclusion appears to be that determination of total IgE as well as specific IgE/total IgE is less predictive than the determination of specific IgE alone, probably as a result of the age-dependent total IgE level and the wide overlap in level between atopic and non-atopic individuals [164;166;167].

Antibody avidity is the overall strength of binding between multivalent antibodies and the antigen for which they are specific. The role of IgE avidity in food allergy remains generally unresolved. El-Khouly *et al*. [165] showed in a study investigating the antibody avidity characteristics of peanut allergic individuals that the peanut allergen Ara h 2-specific IgE avidity correlated with the severity of the allergic disease. Likewise Wang *et al*. [168] reported that high affinity of IgE determined by binding to small peptides was associated with severity of milk allergy. No clear relationship between IgE avidity and level of specific IgE appears to exist [169;170].

Individuals allergic to a specific food may react with great heterogeneity to the different allergens present in that particular food. For example specific IgE from patients with peanut allergy have been shown to bind with considerable heterogeneity to the numerous peanut allergens [171;172]. A reacting pattern of great heterogeneity has been reported to correlate with the severity of the allergic disease. More diverse binding to the different peanut allergens was associated with an increased likelihood of developing severe allergic reaction upon ingestion of peanut, and that diversity was more important than the recognition of specific proteins [160;173].

A study by Christensen *et al*. [174] confirmed that several characteristics of the IgE repertoire may contribute to the functionality of the IgE mediated allergic response. In an *in vitro* test, based on BAT, they showed that the concentration of total IgE, the specific IgE/total IgE ratio, the affinity and clonality (epitope heterogeneity) all affected the degranulation response of the basophils. Individual properties of the IgE repertoire affected different aspects of the degranulation response of basophils (Figure 5), and showed that (1) higher total IgE concentration increased both the sensitivity and maximal degranulation level of the basophils, (2) higher ratios of specific IgE/total IgE increased both the sensitivity and the maximal degranulation level of the basophils, (3) increased equality in concentration of different clones of specific IgE increased the maximal degranulation level of the basophils, (4) higher affinity of individually specific IgE increased the sensitivity of the basophils, and (5) higher clonality increased the sensitivity of the basophils [174]. Such results indicate that no single parameter of the IgE repertoire solely determine the degree of the allergic response.

The pathological role of specific IgG in food allergy, if any, has not been fully established. Conflicting results exist in studies comparing the specific IgG concentrations in atopic and non-atopic individuals, though most find a higher level of specific IgG in atopic individuals compared to non-atopic individuals [109;152;175-178]. For example, Dual *et al*. [152] showed that the level of shrimp-specific IgG correlated directly with

shrimp-specific IgE reactivity, indicating that atopic individuals have a higher level of specific IgG than non-atopic individuals. In contrast, de Jong *et al*. [175] found no difference in the level of specific IgG, between peanut allergic patients and control subjects. de Jong *et al*. [175] though found a greater diversity of the IgG recognition pattern of the peanut allergic patients compared to the individuals serving as control subjects. Similarly, a greater number of IgG epitopes was recognised by hen's egg allergic patients compared to control subjects [179]. It is recognised that specific IgG is not a very useful parameter in the diagnosis of food allergy [175;178].

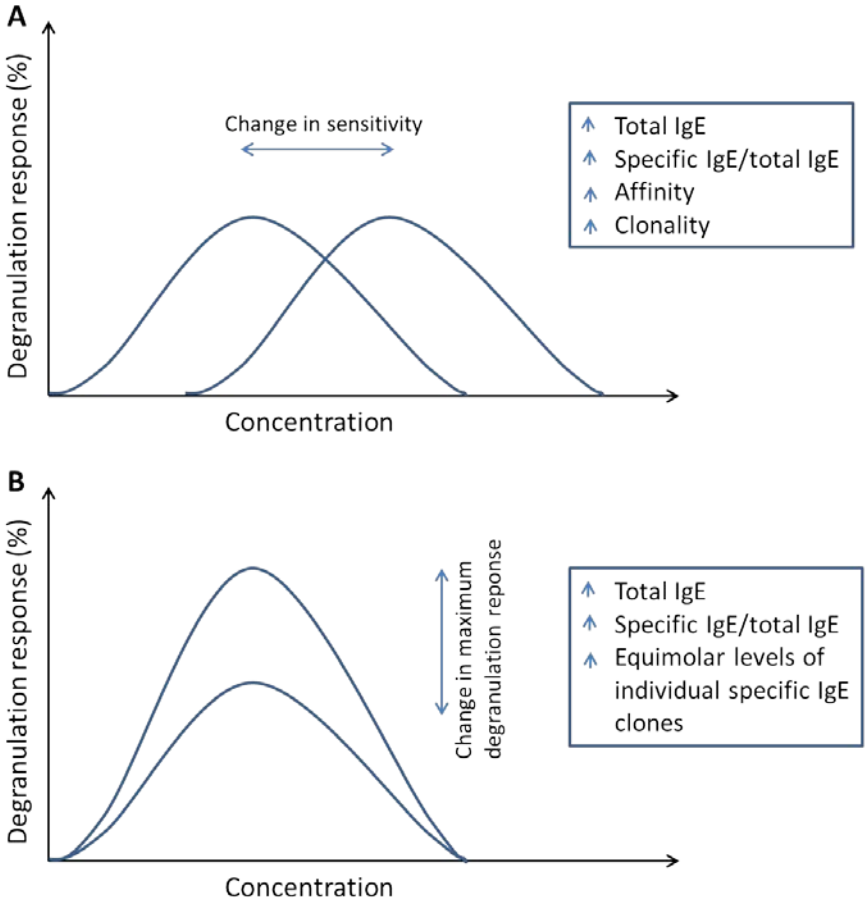

Figure 5. Properties of the IgE repertoire affecting the degranulation response. Different characteristics of the IgE repertoire influences the degranulation response of basophils *in vitro*, by either affecting the sensitivity (the amount of allergen needed for eliciting a half-maximal response) or the level of degranulation response (percentage mediator release of the possible maximum). **A**. Properties of the IgE repertoire affecting the sensitivity. **B**. Properties of the IgE repertoire affecting the maximal degranulation response. Based on Christensen *et al*. [174].

A close relationship have been reported to exist between the specificity of IgE and IgG antibodies [157;177;180-183], where for example Jävinen *et al*. [180] reported that in individuals with persistent cow's milk allergy, the IgG binding epitopes generally colocalised with the IgE binding epitopes and Savilahti *et al*. [157] showed that IgG4 epitopes overlapped with IgE epitopes in cow's milk allergic patients. Colocalisation of IgE and IgG binding epitopes may have great implications for the development of tolerance, since it has been demonstrated that development and maintenance of tolerance could be associated with increased levels of specific IgG4 [156;157;176;184;185], which has been suggested to induce tolerance by blocking the binding of specific IgE to the given food allergen [184;186;187]. Also, high levels of specific IgA have been suggested to correlated with development and maintenance of tolerance [156;185;188;189], though substantial conflicting reports have been presented [109;157]. Recent studies have suggested an implication of free Ig light chain in clinical active type I allergic disease [190-192].

Haddad *et al*. [193] and Selo *et al*. [194;195] showed that cleavage of BLG could result in four different outcomes: (1) complete suppression of IgE binding, (2) reduced IgE binding, (3) no effect on IgE binding, or (4) enhanced IgE binding. This indicates that a great heterogeneity in individual epitope specificity exist, which has also been shown for other milk allergens [159], as well as for peanut allergens [171;177;183].

Recent studies have suggested a particular important role for linear IgE binding epitopes in food allergy. It is suggested that IgE directed towards linear epitopes may react with foods in processed forms (heated and digested), while IgE binding to conformational epitopes may be impaired by such processing because of changes in allergen tertiary structure [4;148]. In addition, linear epitopes have been suggested to be potentially biomarkers for the characterisation of various phenotypes of food allergy [148]. For example, in studies of patients with cow's milk and hen's egg allergy, it was found that those with IgE directed towards linear epitopes in general were more likely to have persistent allergy, while those with IgE directed against conformational epitopes were more likely to develop clinical tolerance [158;159;179;180]. For example, studies with cow's milk allergens [168;180;182;196] and hen's egg allergens [158;179] have suggested an association between linear IgE epitope diversity and persistency. Likewise studies with milk allergens [168], peanut allergens [171;183] and wheat allergens [197] have indicated an association between linear IgE epitope diversity and severity. Shreffler *et al.* [171] found that patients with IgE antibodies recognising many epitopes, identified by IgE binding to overlapping peptides representing the primary sequences of the peanut allergens Ara h 1, Ara h 2 and Ara h 3, tended to develop more severe reactions upon ingestion of peanut compared to those who recognised few epitopes. Wang *et al*. [168] found the same pattern for the milk allergens α_{s1}-, α_{s2}- and κ-caseins as well as for BLG. In contrast, Heinzmann *et al.* [198] reported that the recognition pattern of linear IgE binding epitopes did not vary with the severity of cow's milk allergy. The latter study was only based on the presence of specific IgE antibodies together with the clinical history of cow's milk allergy, which in general make interpretation of the correlation between severity and epitope recognition difficult.

Some of the identified linear IgE binding epitopes have been identified as candidate biomarkers for persistency and severity [158;159;172;182;196;199;200]. For example, Beyer *et al.* [172] reported that peanut allergic patients with persistent allergy recognised specific IgE epitopes, identified by recognition of immunodominant decapeptides, which was not recognised by patient who outgrew their allergy or were peanut sensitised but tolerated peanut ingestion. Järvinen *et al*. [196] identified five specific IgE binding epitopes on milk allergens as informative and reported that IgE recognition of one to three specific epitopes was indicative of persistent cow's milk allergy, a study which was also based on IgE binding to selected decapeptides.

In a study examining the effect of digestion on the allergenicity of kiwifruit proteins, patients with severe allergic reactions recognised epitopes which were resistant to digestion and epitopes emerging as a result of the digestion process, not accessible prior to digestion. Patients with mild reactions recognised digestion labile epitopes. It was suggested that recognition of epitopes developed from the digestion process could be an indicator of a severe allergic phenotype [201]. Likewise Takagi *et al*. [202] found that patients who could recognise pepsinolysis products from the hen's egg allergen ovomucoid (OVM) were more likely not to outgrow their allergy than patients who did not recognise these digestion fragments, which is in agreement with results from a study by Urisu *et al*. [203]. Such results together indicate that combining digestion studies with epitope recognition patterns could be a useful tool for the prediction and diagnosis of the allergic phenotype.

The above mentioned papers generally used methods only allowing identification of linear epitopes. Other studies which in addition to linear epitopes also focused on identification of conformational epitopes have indicated conformational epitopes to be of importance in food allergy [204-207]. This suggest that use of

overlapping short peptides resembling the primary sequence of an allergen for epitope identification, may not be ideal for all dietary proteins.
Although there are some limitations with methods identifying IgE binding epitopes, IgE epitope pattern recognition identification is suggested as being a promising future tool in predicting, diagnosing and treating food allergic individuals [4;148], and may add new knowledge to the understanding of the mechanisms underlying tolerance versus sensitisation [4;148].

Resistance to digestion - a food allergen characteristic?

One of the features proposed to be a characteristic shared by food allergens, which is also one of those that have received most attention in recent years, is resistance to digestion. Stability to proteolysis is generally believed to be an important feature of 'complete' food allergens because a protein able to sensitise the mucosal immune system of the GI tract must survive the digestion process as an intact protein or as large fragments [15-18]. This hypothesis seems highly reasonable, since the longer a significant portion of the protein resists digestion, the more likely it is to encounter the cells of the inductive mucosal immune system and further the more likely it is to retain adequate structure and size to be recognised by these cells. This increases the likelihood of sensitisation and upon reexposure to the allergen increases the likelihood for cross-linking of IgE molecules bound to the surface of effector cells, thereby eliciting allergic responses [15;17]. Studies where impairment of the digestion process led to increased allergenicity of the proteins under investigation further support the hypothesis [57;208;209]. For examples, studies with antacid drugs, medications that hinder peptic digestion by raising pH, converted normally degradable dietary proteins into potentially allergens, promoting sensitisation to caviar proteins in mice [210] and hazelnut proteins in mice and humans [211].

There is no evidence for a specific molecular weight (MW) above which peptides may behave as 'complete' allergens and below which they may not. Still many different suggestions for a lower MW limit, representing the minimum size of a peptide with inherent allergenicity, have been presented. Overall, the general opinion appears to be that the lower limit for allergenicity of peptides is a MW of approximately 3.5 kDa [14;19;212;213]. Such suggestion may, in addition to experimental evidence, be based on the assumption that there must be at least two IgE binding sites on a peptide [14], each constituting a minimum of 15 amino acids covered by the antibody paratope [133]. This equals a minimum of 30 amino acid residues necessary for cross-linking of two IgE molecules and elicitation of an allergic response, and corresponds to an average MW of 3.5 kDa.

In 1996 Astwood *et al*. [18] compared resistance to pepsin digestion in simulated gastric fluid (SGF) for allergenic and non-allergenic proteins. This study was a systematically evaluation of the stability of known food allergens and proteins with no proven allergenicity using a simple *in vitro* model of gastric digestion. The study showed that while major food allergens in general resisted the digestion process, non-allergenic proteins were in contrast rapidly digested. These results were in agreement with the hypothesis that it is a requisite for food allergens to survive the GI digestion process for retainment of sufficient structure and size for uptake and sensitisation when reaching the inductive immune system of the intestinal mucosa. Thereby Astwood *et al*. [18] concluded that stability to digestion is a significant and valid parameter for distinguishing food allergens from non-allergenic dietary proteins and suggested that the ability of food allergens to reach the intestinal mucosa in intact form is a necessity for allergenicity.

The study by Astwood *et al*. [18] contributed to the inclusion of pepsin resistance as a test parameter in the safety assessment of novel proteins in genetically modified foods. In both the report of FAO/WHO from 2001 [19] and the report of Codex Alimentarius Commission from 2003 [21], concerning evaluation of allergenic potential of genetically modified foods, it is recommended that resistance of the novel protein to degradation in the presence of pepsin should be incorporated in the test regime. FAO/WHO [19] recommends that the purified protein in non-heated and non-processed from, as well as the main edible form, should be subjected to pepsin degradation. The pepsin-protein mixture should be prepared using 0.5 mg of protein in 0.2 mL of 0.32% pepsin (w:v), corresponding to a pepsin to protein ratio of 12.8, in 30 mM/L NaCl, pH 2.0, and maintained shaking at 37 °C for 60 min. Aliquots from the digestion mixture should be taken at different time points, at 0, 15 and 30 sec and at 1, 2, 4, 8, 15 and 60 min. Analysis for protein stability and presence of intact protein or intact peptide fragments greater than 3.5 kDa should be evaluated using 10-20% gradient Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels or equivalent gel systems under both reducing and non-reducing conditions, followed by staining procedures (Figure 6) [19]. It is well recognised that the described digestion protocol do not mimic the physiological conditions of the human gastric digestion [21], wherefore the EFSA report from 2010 [20], concerning assessment of allergenicity of genetically modified organisms, recommends that resistance to digestion of novel proteins should be evaluated using other *in vitro* digestibility methods, methods designed to mimic physiological conditions and thereby simulating the conditions of the human digestion process.

Since the study by Astwood *et al*. [18], further studies comparing the digestibility of allergens with non-allergenic dietary proteins have been performed [56;120;214-217]. These studies did in general not support an obvious correlation between resistance to digestion and allergenicity. In contrast to the study by Astwood *et al*. [18], where most of the tested allergens were storage proteins, while all tested protein with no proven allergenicity were enzymes, Fu *et al*. [120] compared allergens with non-allergenic proteins of similar functions. Fu *et al*. [120] showed that some proteins, such as storage proteins were inherently more stable to digestion than other proteins, such as enzymes, and concluded that food allergens were not always more resistant to digestion measured *in vitro* than non-allergenic proteins and that it could be difficult to rank the allergenic potential of proteins based on their susceptibility to pepsin digestion. Kenna and Evans [214] likewise concluded that allergens and proteins with no allergenicity display similar stability to pepsin digestion in SGF and that resistance to digestion is not a characteristic equal to food protein allergenic potential.
While the studies by Astwood *et al*., [18], Fu *et al*., [120], Kenna and Evans [214] and Thomas *et al*. [56] used the visual time disappearances of intact protein and/or large fragments in SDS-PAGE gels as the parameter to assess digestion (which may be a highly subjective parameter for evaluation [217]), Herman *et al*. [216] calculated the half-life of the test protein, assuming a negative exponential (first-order kinetics) decline and Ofori-Anti *et al*. [217] estimated the time point achieving 90% digestion. Ofori-Anti *et al*. [217] report that all three methods have limitations. While the method by Astwood *et al*. [18], Fu *et al*. [120], Kenna and Evans [214] and Thomas *et al*. [56] rely on the reliable detection of bands with residual intact protein or fragments hereof, the methods by Herman *et al*. [216] and Ofori-Anti *et al*. [217] may be difficult to interpret for very labile proteins as well as very stable proteins.

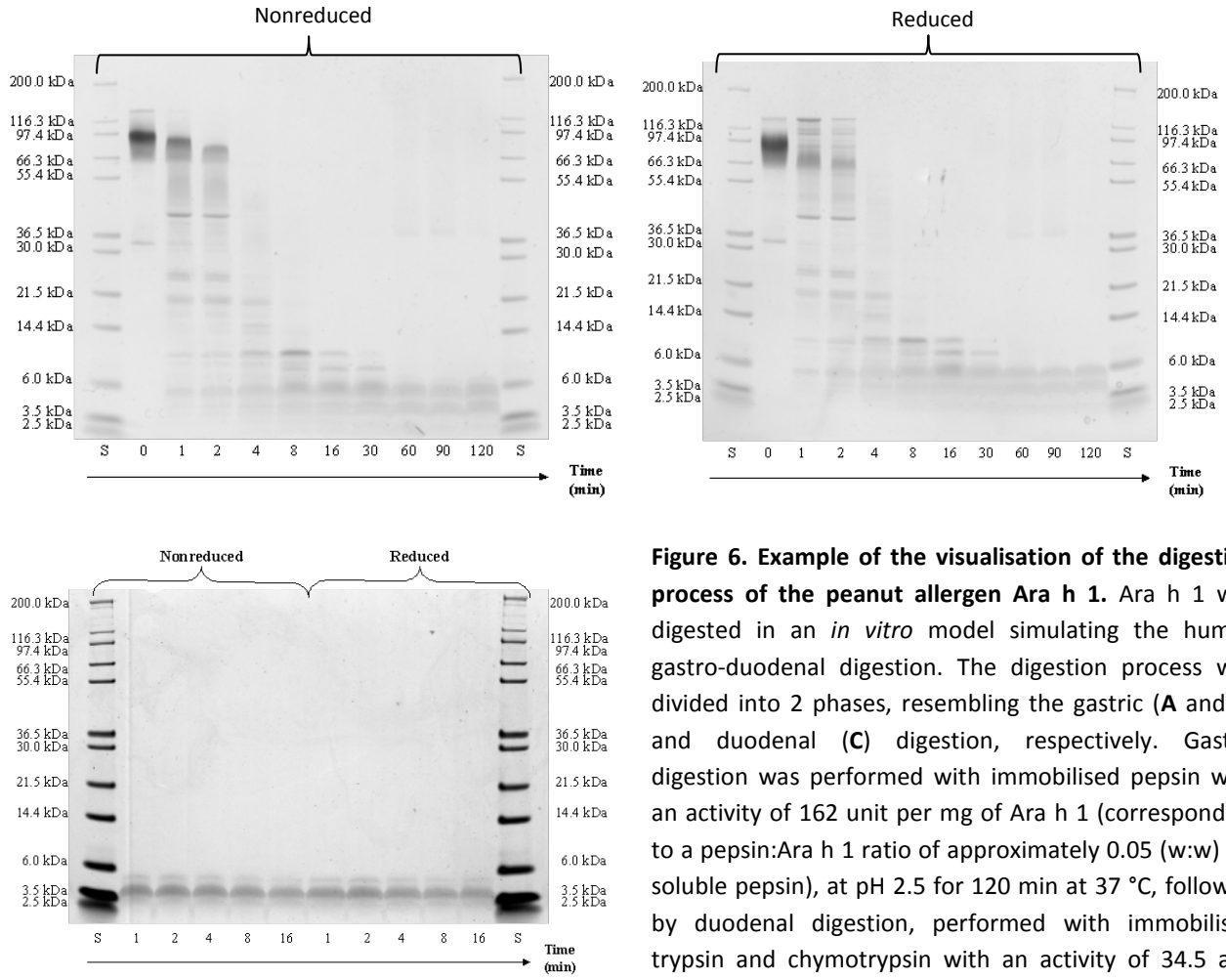

Figure 6. Example of the visualisation of the digestion process of the peanut allergen Ara h 1. Ara h 1 was digested in an *in vitro* model simulating the human gastro-duodenal digestion. The digestion process was divided into 2 phases, resembling the gastric (**A** and **B**) and duodenal (**C**) digestion, respectively. Gastric digestion was performed with immobilised pepsin with an activity of 162 unit per mg of Ara h 1 (corresponding to a pepsin:Ara h 1 ratio of approximately 0.05 (w:w) for soluble pepsin), at pH 2.5 for 120 min at 37 °C, followed by duodenal digestion, performed with immobilised trypsin and chymotrypsin with an activity of 34.5 and 0.44 unit per mg of Ara h 1, respectively, (corresponding to a trypsin:chymotrypsin:Ara h 1 ratio of approximately 0.0025:0.01:1 (w:w:w) for soluble enzymes) at pH 6.5 for 16 min at 37 °C. Aliquots of 10 µL from the gastric digestion process were taken at the time points of 0, 1, 2, 4, 8, 16, 30, 60, 90 and 120 min and from the duodenal digestion process at the time points of 0, 2, 4, 8 and 16 min. SDS-PAGE was performed with 4-12% Bis-Tris Gel with MES running buffer under non- as well as reducing conditions. Gels were stained with SimpleBlue™ (SimpleBlue™ Safestain (Ready-to-use, fast, sensitive and safe Coomassie® G-250) Invitrogen, Carlsbad, CA, USA). As standard markers Mark 12™ was used (Unstained standard, Invitrogen, Carlsbad, CA, USA). [Own unpublished data].

Degradation of known food allergens

Besides comparative studies of the susceptibility to pepsinolysis of known allergens with proteins of no proven allergenicity, many studies have been performed, selectively studying the resistance of known food allergens to pepsin degradation. Table 1 summarises the pepsin stability data of purified food allergens originating from plants and Table 2 summarises the pepsin stability data of purified food allergens originating from animals. While studies of the susceptibility to pepsinolysis is simply used as a model system for evaluation of the general biochemical stability of proteins, or is designed only to mimic the digestion process that takes place in the stomach, several studies have been performed mimicking the physiologic digestion process that takes place in the duodenum as well, as recommended by the EFSA panel [20]. Digestion products from such studies reflect to a greater extent the peptide profile to which the inductive mucosal immune system is exposed, and thereby the situation of oral sensitisation [20]. Table 3 summarises the stability data of purified food allergens exposed to simulated gastro-duodenal digestion.

Digestion conditions, known to influence the outcome of the digestibility assay, such as enzyme to allergen ratios, pH and digestion time, are included in the Tables. Studies of food allergen stability have been performed based on either enzyme mass to allergen mass ratios or enzyme mass to allergen molarity. Only enzyme mass to allergen mass ratios are included in the Table 1-3, based on the statement of Ofori-Anti *et al*. [217] that the use of a constant mass provides the most reliable comparison parameters. Enzyme activity unit per mg of allergen have also been included in the Tables, since different enzyme products can be purchased, as well as there may be batch to batch variation of individual products, with varying activity units per mg of solid enzyme. In addition, studies have been performed using enzymes immobilised to agarose.

No clear definition seems to exist, defining an allergen as labile or stable to digestion. Yet, Goodman and Hefle [218] describe a protein no longer detectable after 2 min of digestion as unstable, a protein no longer detectable between 2 and 15 min of digestion as partially stable, while a protein still detectable after 60 min of digestion is described as stable. In Tables 1-3, an allergen is defined as stable, under the given digestion conditions, when identifiable residual intact protein was left after termination of the digestion process. Stability time varied greatly among the allergens as well as did the stability time for individual allergens under different digestion conditions.

Also no general consensus seems to exist about the importance of the stability of peptide fragments emerging from the proteolysis processes. While all studies on food allergen digestibility evaluated the stability of intact allergen, far less considered the stability of peptide fragments generated during the digestion process, and even less identified the size of the largest fragments (Tables 1-3). However, if the general assumption is that any peptide fragments larger than 3.5 kDa may be of importance for the potential allergenicity of a food allergen [14;19;212;213], although it can not necessarily be ranked alongside the intact protein, greater effort for identifying such fragments may be reasonable. Thomas *et al*. [56], Herman *et al*. [216], Takagi *et al*. [215] as well as Ofori-Anti *et al*. [217] all acknowledged the importance of considering stable peptide fragments derived from food allergens that are rapidly digested, such as the major peanut allergens Ara h 1 and Ara h 2 as well as the major hen's egg allergen OVM, which form stable fragments upon digestion that retain significant IgE binding capacities [203;219-221]. In addition, the EFSA panel [20] expresses that the appearance of stable peptide fragment or aggregates hereof formed during the digestibility assay should be considered as a risk factor for allergenicity, and recommends that peptide fragments and aggregates should be further investigated. Thus in addition to the stability of the intact proteins, the stability of the peptide fragments formed during the digestion process, also needs to be evaluated [20].

Table 1. Stability of allergens derived from plant foods to digestion with pepsin[a].

Source	Allergen[b]	Size[c] (kDa)	Pepsin: aller-gen MW ratio	Enzyme activity unit/mg allergen	pH	Diges -tion time (min)	Stabi-lity	Stabi-lity time[d] (min)	Largest frag-ments (kDa)	Ref.
Peanut	Ara h 1, Cupin (Vicilin-type, 7S globulin)	64	nd[e]	162	2.5	120	no	4	4	*
			0.05	162	2.5	120	no	1	~5.6	[222]
			12.8	nd	1.2	120	no	5	nd	[120]
			3.04	10,000	1.2	60	no	0.5	nd	[223]
			0.30	1,000	1.2	60	no	0.5	nd	[223]
			0.03	100	1.2	60	no	0.5	nd	[223]

Table 1. Stability of allergens derived from plant foods to digestion with pepsin[a]. Continued….

Source	Allergen[b]	Size[c] (kDa)	Pepsin: aller-gen MW ratio	Enzyme activity unit/mg allergen	pH	Diges-tion time (min)	Stabi-lity	Stabi-lity time[d] (min)	Largest frag-ments (kDa)	Ref.
Peanut	Ara h 2, Conglutin (2S albumin)	17	3	10,000	1.2	60	no	0-2	~10	[56]
			3	10,000	2	60	no	0-30	~10	[56]
			19	nd	1.2	60	yes	-	-	[18]
			12.8	nd	1.2	120	no	0.5	nd	[120]
			3.04	10,000	1.2	60	no	16	~10	[223]
			0.30	1,000	1.2	60	yes	-	-	[223]
			0.03	100	1.2	60	yes	-	-	[223]
			0.4	nd	2.1	40	no	-	-	[220]
	Ara h 3, Cupin (Legumin-type, 11S globulin, Glycinin)	60	3.04	10,000	1.2	60	no	0.25	nd	[223]
			0.30	1,000	1.2	60	no	0.25	nd	[223]
			0.03	100	1.2	60	no	0.25	nd	[223]
			0.002	nd	2	120	no	<10	<14	[224]
	Ara h 6 Conglutin (2S albumin)	15	3.04	10,000	1.2	60	no	4	~10	[223]
			0.30	1,000	1.2	60	no	16	~10	[223]
			0.03	100	1.2	60	yes	-	-	[223]
			nd[e]	6.49	3	120	yes	-	-	[225]
	Lectin?	?	19	Nd	1.2	60	no	8	non visual	[18]
			12.8	nd	1.2	120	no	5	nd	[120]
Soy	Gly m 1 (ns-LTP)	7	19	nd	1.2	60	no	0	non visual	[18]
			12.8	nd	1.2	120	no	2	nd	[120]
	Gly m 5, Conglycinin (Vicilin-type, 7S globulin) α-subunit	67	19	nd	1.2	60	no	2	non visual	[18]
			12.8	nd	1.2	120	no	0	nd	[120]
	Gly m 5, Conglycinin, (Vicilin-type, 7S globulin) β-subunit	48	19	nd	1.2	60	yes	-	-	[18]
			12.8	nd	1.2	120	yes	-	-	[120]
	Gly m 6, Glycinin (Legumin-type, 11 S globulin)	60	0.002	nd	2	120	no	-	<25	[224]
	STI, Trypsin-inhibitor?	19	3	10,000	1.2	60	yes	-	-	[56]
			3	10,000	2	60	yes	-	-	[56]
			19	nd	1.2	60	yes	-	-	[18]
			12.8	nd	1.2	120	yes	-	-	[120]
		19	3.04	10,518	2	60	yes	-	-	[215]
			0.94	nd	1.2	60	yes	-	-	[226]
	Lectin?	?	19	nd	1.2	60	no	15	non visual	[18]
			12.8	nd	1.2	120	no	5	nd	[120]
Black gram	?	28	0.94	nd	1.2	60	no	15	~16	[227]

Table 1. Stability of allergens derived from plant foods to digestion with pepsin[a]. Continued…

Source	Allergen[b]	Size[c] (kDa)	Pepsin: aller-gen MW ratio	Enzyme activity unit/mg allergen	pH	Diges -tion time (min)	Stabi-lity	Stabi-lity time[d] (min)	Largest frag-ments (kDa)	Ref.
Brazil nut	Ber e 1, (2S albumin)	9+4	16.7	nd	1.2	60	no	15	nd	[228]
			0.05	182	2.5	120	yes	-	-	[54]
			0.05[f]	182	2.5	120	yes	-	-	[54]
			1.3	9,600	2	60	yes	-	-	[229]
			16.7	nd	1.2	60	no	15	nd	[230]
Hazelnut	Cor a 1, PR-10 (Bet v 1 homologue[g])	17.5	0.4	nd	1	30	no	0	~1.6	[117]
Sesame seed	Ses i 1, (2S albumin)	9+4	0.05	182	2.5	120	yes	-	-	[231]
			0.05[f]	182	2.5	120	yes	-	-	[231]
			0.05[h]	182	2.5	120	yes	-	-	[231]
Sunflower seed	SFA-8, (2S albumin)	?	16.7	nd	1.2	60	no	30	nd	[228]
			16.7	nd	1.2	60	no	5	nd	[230]
Rapeseed	Bra n 1, (2S albumin)	9+4	16	nd	1.2	60	yes	-	-	[232]
Rice	?	~16	0.025	nd	1.2	120	yes	-	-	[233]
Yellow mustard	Sin a 1, (2S albumin)	9+4	19	nd	1.2	60	yes	-	-	[18]
Oriental mustard	Bra j 1, (2S albumin)	9+4	19	nd	1.2	60	yes	-	-	[18]
Potato	Sola t 1 (Phatatin)	43	12.8	nd	1.2	120	no	0	nd	[120]
Celery	Api g 1, PR-10 (Bet v 1 homologue[g])	15	0.43	nd	1	30	no	0	~1.2	[117]
	Api g 2, (ns-LTP)	10	0.05	nd	2	120	yes	-	-	[234]
Peach	Pru p 3, (ns-LTP)	10	20	nd	1.2	30	yes	-	-	[235]
			6.4	nd	1.2	30	yes	-	-	[236]
			0.017	nd	2	180	yes	-	-	[237]
Grape	Vit v 1, (ns-LTP)	9	0.05	182	2.5	120	yes	-	-	[43]
			0.05[f]	182	2.5	120	yes	-	-	[43]
Cherry	Pru av 1, PR-10 (Bet v 1 homologue[g])	9	nd[e]	≥60,000	2.5	120	no	30	nd	[116]
	Pru av 3, (ns-LTP)	10	nd[e]	≥60,000	2.5	120	yes	-	-	[116]
	Pru av 4, (Profilin[g])	15	nd[e]	≥60,000	2.5	120	no	1	nd	[116]
Apple	Mal d 1, PR-10 (Bet v 1 homologous[g])	17.7	1.74	nd	2	30	no	0	nd	[238]
			0.4	nd	1	30	no	0	~1.8	[117]
Melon	Cuc m 2, (Profilin[g])	14	6.4	nd	1.2	30	no	<30	non visual	[236]
Papaya	Papain superfamily	?	12.8	nd	1.2	120	no	0	nd	[120]
Pineapple	Bromelain, papain superfamily	?	12.8	nd	1.2	120	no	0	nd	[120]

Table 1. Stability of allergens derived from plant foods to digestion with pepsin[a]. Continued…

Source	Allergen[b]	Size[c] (kDa)	Pepsin: allergen MW ratio	Enzyme activity unit/mg allergen	pH	Digestion time (min)	Stability	Stability time[d] (min)	Largest fragments (kDa)	Ref.
Kiwi	Act c 1, actinidin	27.4	0.05	212	2.5	60	yes	-	-	[239]
	Act c 2, taumatin-like protein	24	0.05	212	2.5	120	yes	-	-	[239]
			6.5	nd	1.2	60	no			[50]
			16	nd	1.2	60	no	1	nd	[240]
	Actinidin, papain superfamily	?	12.8	nd	1.2	120	no	0	nd	[120]
Avocado	Pers a 1, chitinase[g]	32	20	nd	1.2	30	no	0	~5-6	[235]

[a]Summary of allergen stability to pepsin, simulating the digestion process that takes place in the stomach. Included in the Table is only digestibility results from pure plant food derived allergens, digested at 37 °C, for which it was possible to identify a pepsin to allergen ratio, either expressed by an MW ratio or an enzyme activity per mg allergen ratio, [b]Allergen name and structural family to which it belong are based on the Allergen nomenclature (IUIS Allergen Nomenclature Sub-Committee). www.allergen.org. [c]Sizes of allergens are based on the Allergen nomenclature (IUIS Allergen Nomenclature Sub-Committee). www.allergen.org, [d]Stability time is based on either the one described by the given author or from the visual appearance in a presented SDS-PAGE [e]Immobilised enzymes, [f]Surfactant, [g]Non-sensitising allergen, [h]Preheating, [*]Own unpublished data, Abbreviations: MW, molecular weight; nd, not described; ns-LTP, nonspecific-lipid transfer protein; PR-10, pathogenesis-related protein-10; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Table 2. Stability of allergens derived from animal foods to digestion with pepsin[a].

Source	Allergen[b]	Size[c] (kDa)	Pepsin: allergen MW ratio	Enzyme activity unit/mg allergen	pH	Digestion time (min)	Stability	Stability time[d] (min)	Largest fragments (kDa)	Ref.
Cow's milk	Bos d 4 (ALA)	14.2	12.8	nd	1.2	120	no	0	nd	[120]
			0.02	nd	2	90	no	nd	~7.5	[241]
			0.02	nd	3	90	no	nd	~7.5	[241]
			0.02	nd	4	90	yes	-	-	[241]
			0.05	182	2.5	120	no	5	< 6.5	[41]
			0.05[e]	182	2.5	120	no	30	< 6.5	[41]
	Bos d 5 (BLG)	18.3	3	10,000	1.2	60	yes	-	-	[56]
			3	10,000	2	60	yes	-	-	[56]
			0.05[e]	182	2.5	60	yes	-	-	[39]
			0.007[e]	25	3	60	yes	-	-	[39]
			19	nd	1.2	60	yes	-	-	[18]
			12.8	nd	1.2	120	yes	-	-	[120]
			3.04	10,518	2	60	yes	-	-	[215]
			0.02	nd	2	90	yes	-	-	[241]
			0.02	nd	3	90	yes	-	-	[241]
			0.02	nd	4	90	yes	-	-	[241]
			0.05	182	2.5	60	yes	-	-	[242]
			0.05	182	2.5	60	yes	-	-	[42]
			12.8	42,240	1.2	120	no	0	nd	[243]
			0.05	165	2.5	60	yes	-	-	[243]

Table 2. Stability of allergens derived from animal foods to digestion with pepsin[a]. Continued…

Source	Allergen[b]	Size[c] (kDa)	Pepsin: aller-gen MW ratio	Enzyme activity unit/mg allergen	pH	Diges -tion time (min)	Stabi-lity	Stabi-lity time[d] (min)	Largest frag-ments (kDa)	Ref.
	Bos d 5, (BLG)		0.05[e]	165	2.5	60	yes	-	-	[243]
			3.05	10,000	1.2	60	yes	-	-	[217]
			0.94	nd	1.2	60	yes	-	-	[226]
			nd	10,000	1.5	60	yes	-	-	[201]
			16	55,356	1.2	60	yes	-	-	[244]
	Bos d 6, (BSA)	37	3	10,000	1.2	60	no	0-2	~6	[56]
			3	10,000	2	60	no	0-2	~6	[56]
			19	nd	1.2	60	no	0.5	non visual	[18]
			3.05	10,000	1.2	60	no	0.5	~3.9	[217]
			12.8	nd	1.2	120	no	0	nd	[120]
			0.02	nd	2	90	no	nd	~11	[241]
			0.02	nd	3	90	no	nd	~11	[241]
			0.02	nd	4	90	no	nd	~31	[241]
			3.04	10,518	2	60	no	0.5	~3.9	[215]
			13	nd	1.2	60	no	5	nd	[50]
			0.025	nd	1.2	120	no	<10	non visual	[233]
			16.7	nd	1.2	60	no	5	nd	[230]
			16	nd	1.2	60	no	0	nd	[232]
			16.7	nd	1.2	60	no	0	nd	[228]
	Bos d 7, (IgG)	160	0.02	nd	2	90	no	nd	~30	[241]
			0.02	nd	3	90	yes	-	-	[241]
			0.02	nd	4	90	yes	-	-	[241]
	Bos d 8, (β-casein)	24	0.007[e]	25	3	60	no	10	7.4	[39]
			19	nd	1.2	60	no	2	non visual	[18]
			12.8	42,240	1.2	120	no	0.1	nd	[243]
			0.05	165	2.5	60	no	10	≥3.5	[243]
			0.05[e]	165	2.5	60	no	10	≥3.5	[243]
			0.05	182	2.5	60	no	20	nd	[242]
	Bos d 8, (α-casein)	?	12.8	nd	1.2	120	no	0	nd	[120]
	Lactoperoxidase	?	12.8	nd	1.2	120	no	0	nd	[120]
	Lactoferrin	?	3.05	10,000	1.2	60	no	0.5	~5.4	[217]
Hen's egg	Gal d 1, (OVM)	28	19	nd	1.2	60	no	8	non visual	[18]
			12.8	nd	1.2	120	no	0	nd	[120]
			3.04	10,518	2	60	no	0.5	<3	[215]
			nd	10,000	2	60	no	0.5	4.5-6	[202]
			nd	10,000	2	60	no	2	4.5-6	[202]
			nd	10,000	2	60	no	30	4.5-6	[202]
			0.05	nd	2	120	no	10	18	[245]
			0.05	nd	2.5	60	no	10	18	[246]
			0.05[f]	nd	2.5	60	no	10	18	[246]
	Gal d 2, (OVA)	44	3	10,000	1.2	60	yes	-	-	[56]
			3	10,000	2	60	yes	-	-	[56]
			0.05	182	2.5	60	yes	-	-	[39]
			0.007	25	3	60	yes	-	-	[39]

Table 2. Stability of allergens derived from animal foods to digestion with pepsin[a]. Continued…

Source	Allergen[b]	Size[c] (kDa)	Pepsin: aller-gen MW ratio	Enzyme activity unit/mg allergen	pH	Diges tion time (min)	Stabi-lity	Stabi-lity time[d] (min)	Largest frag-ments (kDa)	Ref.
Hen's egg	Gal d 2 (OVA)		19	nd	1.2	60	yes	-	-	[18]
			12.8	nd	1.2	120	no	5	nd	[120]
			3.04	10,518	2	60	yes	-	-	[215]
			3.04	10,518	2	60	no[d]	0.5	non visual	[215]
			8		1.2	60	yes	-	-	[247]
			2		1.2	60	yes	-	-	[247]
			3.05	10,000	1.2	60	yes	-	-	[217]
			16	55,356	1.2	60	no	15	nd	[244]
			0.2	114	2.5	120	yes	-	-	[248]
			0.05	nd	2.5	60	yes	-	-	[246]
			0.05[f]	nd	2.5	60	no	10	~4	[246]
			0.05	172	2	60	yes	-	-	[249]
			0.05[e]	172	2	60	yes	-	-	[249]
	Gal d 3, (OT)	78	19	nd	1.2	60	no	0	non visual	[18]
			12.8	nd	1.2	120	no	0	nd	[120]
	Gal d 4, (HEL)	14	12.8	nd	1.2	120	no	60	nd	[120]
			3.05	10,000	1.2	60	yes	-	-	[217]
			16	55,356	1.2	60	no	8	nd	[244]
	Phosvitin, kinase?	?	19	nd	1.2	60	yes	-	-	[18]
Fish, whiff	Lep w 1, Parvalbumin	12	0.05[f]	212	2.5	120	yes/ no[g]	0	nd	[250]
Shrimp	Pen a 1, Tropomyosin	36	12.8	nd	1.2	120	no	0	nd	[120]
	Lit v 1, Tropomyosin	36	0.02	333	1.2	60	yes	-	-	[251]
Prawn	Pen m 1, Tropomyosin	38	0.02	333	1.2	60	yes	-	-	[251]
Crab	Tropomyosin	34	0.02	333	1.2	60	yes	-	-	[252]
			0.02	333	1.2	60	yes	-	-	[253]

[a]Summary of allergen stability to pepsin, simulating the digestion process that takes place in the stomach. Included in the Table is only digestibility results from pure animal foods derived allergens, digested at 37 °C, for which it was possible to identify a pepsin to allergen ratio, either expressed by an MW ratio or an enzyme activity per mg allergen ratio, [b]Allergen name and structural family to which it belong are based on the Allergen nomenclature (IUIS Allergen Nomenclature Sub-Committee). www.allergen.org. [c]Sizes of allergens are based on the Allergen nomenclature (IUIS Allergen Nomenclature Sub-Committee). www.allergen.org, [d]Stability time is based on either the one described by the given author or from the visual appearance in a presented SDS-PAGE, [e]Surfactant, [f]Preheating, [g]The protein was stable as a dimer but not as a monomer, Abbreviations: ALA, α-lactalbumin; BLG, *β*-lactoglobulin; BSA, bovine serum albumin; HEL, hen egg lysozyme C; MW, molecular weight; nd, not described; OT, ovotransferrin; OVA, ovalbumin; OVM, ovomucoid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Table 3. Stability of allergens derived from plant and animal foods to digestion with pepsin followed by trypsin and chymotrypsin[a].

Source	Allergen[b]	Size[c] (kDa)	Pepsin/Trypsin/Chymotrypsin:allergen MW ratio	Enzyme activity units/mg allergen	pH	Digestion time (min)	Stability	Stability time[d] (min)	Largest fragments (kDa)	Ref.
Peanut	Ara h 1, Cupin (Vicilin-type 7S globulin)	63.5	nd[e]	162/ 34.5/0.44	2.5/6.5	120+ 16	no	4	2	*
			0.05/ 0.0025/0.01	162/ 34.5/0.44	2.5/6.5	120+ 16	no	1	-	[222]
Brazil nut	Ber e 1, (2S albumin)	9+4	0.05/ 0.0025/0.01	182/ 34.5/0.44	2.5/6.5	120+ 120	no	120+ nd	1.1	[54]
			0.05/ 0.0025/0.01[f]	182/ 34.5/0.44	2.5/6.5	120+ 120	no	120+ nd	1.1	[54]
Sesame seed	Ses i 1, (2S albumin)	9+4	0.05/ 0.0025/0.01	182/ 34.5/0.44	2.5/6.5	120+ 120	no	120+ nd	nd	[231]
			0.05/ 0.0025/0.01[f]	182/ 34.5/0.44	2.5/6.5	120+ 120	no	120+ nd	nd	[231]
			0.05/ 0.0025/0.01[h]	182/ 34.5/0.44	2.5/6.5	120+ 120	no	120+ nd	nd	[231]
Grape	Vit v 1, (ns-LTP)	9	0.05/ 0.0025/0.01	182/ 34.5/0.44	2.5/6.5	120+ 120	yes	-	-	[43]
			0.05/ 0.0025/0.01[f]	182/ 34.5/0.44	2.5/6.5	120+ 120	yes	-	-	[43]
Peach	Pru p 3, (ns-LTP)	10	0.017/ 0.017/0.017	nd	2/7.8	180+ 240	yes	-	-	[237]
Celery	Api g 2, (ns-LTP)	10	0.05/ 0.0025/0.01	nd	2/7.8	120+ 120	yes	-	-	[234]
Kiwi	Act d 1	27.4	0.05/ 0.0025/0.01	212/ 34.5/0.44	2.5/6.5	60+ 30	yes	-	-	[230]
	Act d 2	24	0.05/ 0.0025/0.01	212/ 34.5/0.44	2.5/6.5	120+ 120	yes	-	-	[230]
Apple	Mal d 1[g]	17.7	1.74/ 0.844/0	nd	2/7.7	30+ 30	no	0	nd	[238]
Cow's milk	Bos d 4 (ALA)	14.2	0.05/ 0.0025/0.01	182/ 34.5/0.44	2.5/6.5	120+ 120	no	5	<<6.5	[41]
			0.05/ 0.0025/0.01[f]	182/ 34.5/0.44	2.5/6.5	120+ 120	no	30	<<6.5	[41]
	Bos d 5, (BLG)	18.3	0.05/ 0.0025/0.01[f]	182/ 34.5/0.4	2.5/6.5	60+ 30	yes	-	-	[39]
			0.05/ 0.0025/0.01[f]	182/ 34.5/0.4	3/6.5	60+ 30	yes	-	-	[39]
			0.05/ 0.0025/0.01	165/ 34.5/0.5	2.5/6.5	60+ 30	yes	-	-	[243]
			0.05/ 0.0025/0.01[f]	165/ 34.5/0.4	2.5/6.5	60+ 30	yes	-	-	[243]
			0.05/ 0.0025/0.01	182/ 34.5/0.4	2.5/6.5	60+ 30	yes	-	-	[42]
			0.05/ 0.0025/0.01[f]	182/ 35.5/0.4	2.5/6.5	60+ 30	yes	-	-	[42]
			0.05/ 0.0025/0.01	182/ 34.5/0.4	2.5/6.5	60+ 30	no	60+ 15	nd	[242]
	Bos d 8 (β-casein)	24	0.05/ 0.0025/0.01[f]	182/ 34.5/0.4	2.5/6.5	60+ 30	no	10	~1.73	[39]

Table 3. Stability of allergens derived from plant and animal foods to digestion with pepsin followed by trypsin and chymotrypsin[a]. Continued…

Source	Allergen[b]	Size[c] (kDa)	Pepsin/Trypsin/Chymotrypsin:allergen MW ratio	Enzyme activity units/mg allergen	pH	Digestion time (min)	Stability	Stability time[d] (min)	Largest fragments (kDa)	Ref.
Cow's milk	Bos d 8 (β-casein)		0.05/0.0025/0.01[f]	182/34.5/0.4	3/6.5	60+30	no	10	~3	[39]
			0.05/0.0025/0.01	165/34.5/0.5	2.5/6.5	60+30	no	10	non visual	[243]
			0.05/0.0025/0.01[f]	165/34.5/0.4	2.5/6.5	60+30	no	10	non visual	[243]
			0.05/0.0025/0.01	182/34.5/0.4	2.5/6.5	60+30	no	20	nd	[242]
Hen's egg	Gal d 1 (OVM)	28	0.005/0.002/0.002	nd	2/~7.5	30+180	no	nd	>20	[245]
	Gal d 2 (OVA)	44	0.05/0.0025/0.01[f]	182/34.5/0.4	2.5/6.5	60+30	yes	-	-	[39]
			0.05/0.0025/0.01[f]	182/34.5/0.4	3/6.5	60+30	yes	-	-	[39]
			0.05/nd/nd	172/40/0.5	2/7	60+60	yes	-	-	[249]

[a]Summary of allergen stability to pepsin, simulating the digestion process that takes place in the stomach, followed by trypsin and chymotrypsin, simulating the digestion process that takes place in the duodenum. Included in the Table is only digestibility results from pure plant and animal foods derived allergens, digested at 37 °C, for which it was possible to identify a enzyme to allergen ratios, either expressed by an MW ratio or an enzyme activity per mg allergen ratio, [b]Allergen name and structural family to which it belong are based on the Allergen nomenclature (IUIS Allergen Nomenclature Sub-Committee). www.allergen.org. [c]Sizes of allergens are based on the Allergen nomenclature (IUIS Allergen Nomenclature Sub-Committee). www.allergen.org, [d]Stability time is based on either the one described by the given author or from the visual appearance in a presented SDS-PAGE, [e]Immobilised enzymes, [f]Surfactant, [g]Non-sensitising allergen, [h]Preheating, [*]Own unpublished data, Abbreviations: ALA, α-lactalbumin; BLG, *β*-lactoglobulin; MW, molecular weight; nd, not described; ns-LTP, nonspecific-lipid transfer protein; OVA, ovalbumin; OVM, ovomucoid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; STI, soybean trypsin inhibitor.

More than half of the digestibility evaluated allergens, proved no stability to digestion (Table 1-3). For example for the plant derived food allergens 19 proved no stability to pepsinolysis, 5 showed both to be stable and labile, depending on the digestibility conditions, while 11 proved to be stabile to the pepsin digestion (Table 1). This indicates that no strict correlation exist between resistance to digestion and allergenicity. This viewpoint is in agreement with Fu *et al*. [120;254], stating in 2002 that such correlation was not absolute.

Although the exact mechanisms by which food allergens sensitise are currently unknown, food allergens are thought to sensitise through the oral route, and consequently sensitise through the intestinal mucosal immune system [255]. Such food allergens are designated 'complete' allergens [115]. In addition to 'complete' food allergens, some food protein, known to elicit allergic reactions, are thought not to induce allergic sensitisation [255;256]. Patients suffering from e.g. pollen or latex allergies may experience immediate reactions upon ingestion of foods, mainly fruits and vegetable [257]. Such association is due to

cross-reacting IgE antibodies, recognising structures of the dietary protein homologous to IgE binding sites of pollen or latex allergens [257]. In Europe, more than 70% of patients suffering from pollen allergy may experience symptoms after food intake. Here the food allergens from e.g. the pathogenesis-related protein-10 (PR-10) family, Mal d 1 from apple, Cor a 1 from hazelnut, Pru av 1 from cherry and Api g 1 from celery are allergens that cross-react with IgE antibodies specific for the major birch pollen allergen Bet v 1 and e.g. the food allergens from the profiling family such as Pru av 4 from cherry is an allergen cross-reacting with the birch pollen allergen Bet v 2 [257]. All five allergens are proteins labile to pepsinolysis (Table 1) [116;117] as well are other dietary proteins considered to be non-sensitising allergens [201;226;258]. These non-sensitising allergens are usually associated with oral allergy syndrome (OAS), which is mild allergic reactions restricted to the oral cavity [256;257]. An assessment of the stability to digestion may therefore not be relevant for such 'incomplete' food allergens and indicate that an *in vitro* digestibility assay for assessment of potential allergenicity could be misleading for allergens of non-sensitising characteristic, eliciting allergic reactions based on cross-reaction with IgE antibodies raised against other non-food allergens.

Taking into account only food allergens believed to sensitise through the intestinal mucosa, data from digestibility assays indicate that there are still more 'complete' food allergens that are susceptible to digestion than those completely stable. For example the major peanut allergen Ara h 1 and the major cow's milk allergen *β*-casein have been shown to be highly labile proteins, braking down to small peptide fragments within minutes [222;223;243;259]. Hence for dietary proteins sensitising through the intestinal mucosal no significant correlation between resistance to digestion and allergenicity seems to exist.

Susceptibility of food allergens to proteolysis has mostly been tested by analysing the effects of pepsinolysis. Comparing Table 1 and 2 with Table 3 reveals that some allergens, such as the major allergen Ber e 1 from Brazil nut may be resistant to pepsinolysis but on the other hand, be broken down in a process simulating the gastro-duodenal digestion [54], where the protein in addition to pepsinolysis also is proteolysed by trypsin and chymotrypsin. In addition, Takagi *et al*. [215] showed that some food allergens, such as the major cow's milk allergen BLG was resistant to pepsinolysis in SGF and labile to digestion by pancreatin in simulated intestinal fluid (SIF). In contrast, other known allergens, such as the cow's milk allergen BSA were labile to digestion in SGF, while resistant to digestion in SIF. Such results, in agreement with the perception of the EFSA panel [20], indicate the usefulness of not only implementing a degradation phase resembling the gastric digestion process but also a phase resembling the duodenal digestion process. This will achieve a more thorough knowledge of the general stability of the dietary protein, and get a more realistic picture of the digestion profile that the intestinal immune system may be exposed to. This is also in accordance with the message by Moreno [255] pointing out the importance of using physiologically relevant *in vitro* digestion, simulating the gastric as well as the intestinal digestion process.

Factors influencing the outcome of the digestibility assay

Extensive variability in assay condition and hence the resulting outcomes exist among the different digestibility studies (Table 1-3). For example the stability of Ara h 2 (Table 1) to pepsinolysis under different conditions resulted in either Ara h 2 being an easily digestible protein [56;120] or being a highly resistant protein [18;223]. Similar results were shown for the peanut allergen Ara h 6 [223;225] and the Brazil nut allergen Ber e 1 [54;228-230]. Also the milk allergen BLG, which is normally regarded as a protein highly resistant to pepsin digestion [18;39;42;56;120;215] was in a single study found to be susceptible to pepsinolysis [243] and in another study found to be susceptible to simulated gastro-duodenal digestion [242]. Comparison of such studies reveals the very importance of the assay condition used, and points to

the necessity for using the same assay condition for comparability between studies and the prerequisite to decide the assay condition most suitable for the assessment of allergenic potential of novel proteins.
In addition, two separate studies using the same assay conditions, showed contradicting results, where Mandalari *et al*. [243] found BLG to be degraded by pepsin digestion, and Fu *et al*. [120] found BLG to be stable to the pepsinolysis. This indicates inter-laboratory differences, and points to the necessity of using standardised assay conditions.
Factors such as digestion time, enzyme to allergen ratio, pH, purity of allergen, allergen processing, presence of surfactant and presence of food matrix may all have a great impact on the outcome of the digestion assay. The methods used for determination of residual intact protein as well as amount and sizes of emerging peptide fragments may on the other hand have a significant influence on the interpretation of the susceptibility of the given protein to digestion.

Huge variations in protease to allergen ratios exist (Table 1-3). Many studies were performed under the assay condition with an enzyme activity of approximately 10,000 unit per mg of protein, presented by Thomas *et al*. [56]. These studies are far from physiological, based on a standard SGF used for preclinical assessment of pharmaceuticals and only gives a biochemical measure of a protein's overall physiochemical stability [20;243]. Other studies were performed with an enzyme activity of approximately 180 units per mg of protein, presented by Moreno *et al*. [54]. These studies seek to elucidate the role of physiologically relevant digestion on the peptide profile of digestion products, and hence what the intestinal mucosal immune system is exposed to [20;41].
In a study by Takagi *et al*. [202] digestion of OVM at different pepsin to allergen ratios showed that the stability of the intact protein as well as some of the generated peptide fragments increased markedly by lowering the pepsin concentration 10 and 100-fold. In addition, Mandalari *et al*. [243] showed that BLG was resistant to pepsinolysis under physiologically relevant pepsin to allergen ratio, while it was degraded by increasing the pepsin concentration 256-fold. The enzyme to allergens ratio also significantly affected the stability of the peanut allergens Ara h 2 and Ara h 6. Both were shown to be stable at an enzyme activity of 100 unit per mg of peanut protein. They were completely labile by raising the enzyme activity 100-fold [223]. These studies show that the digestibility of allergens is significantly influenced by the enzyme to protein ratio, so that an allergen appearing to be stable at one ratio became susceptible at higher ratios. In addition to the intact protein also the amount and sizes of stable fragment may significantly be affected by the enzyme to protein ratio, which is in agreement with the message by Fu *et al*. [120].

The pH under which the digestion assay is performed also varies (Table 1-3). In studies of the susceptibility of food allergens to digestion in the presence of solely pepsin, the pH varied between 1 and 4. No single pH will ever mimic the condition in the stomach. While the pH of the fasted stomach is around 2 [30-32], the pH rises after the ingestion of food to a pH of up to 5 [20].
Lucas *et al*. [201] digested kiwi allergens at different pH values and showed that the stability of the allergens was significantly affected by the pH where an increase from pH 1.5 to 2.5 significantly reduced proteolytic break down of the allergens and a further increase in pH to 3 abolished the break down. In addition, studies of the digestibility of codfish proteins showed that proteins were degraded within 1 minute at pH ranging from 1.25 to 2.5, while only a marginal shift in pH from 2.5 to 2.75 completely abrogated the digestion of the cod allergens [57]. Also studies of hen's egg allergen digestion showed that digestibility of both OVM, OVA, Ovotransferrin (OT) and hen's egg lysozyme C (HEL) were pH dependent, though to different degrees [209]. These studies show that the digestibility of allergens may greatly be

affected by variation in pH, probably because of the pH-dependent activity of the enzymes as well as the pH dependent denaturation of proteins.

Also addition of surfactant, such as PC and bile salts (which is secreted by the gastric mucosa or being a constituent of the bile, respectively) may greatly affect the susceptibility of proteins to digestion (Tables 1-3). For example, addition of PC altered the kinetics of the BLG degradation, protecting the allergen from digestion by slowing the digestion process as well as altered the pattern of the generated peptide fragments [39;42;242;243]. A protective impact of PC was also seen on the degradation of the cow's milk allergen α-lactalbumin (ALA) [41] as well as the grape allergen Vit v 1 [43]. In contrast PC did not influence the digestibility of the cow's milk allergen *β*-casein [243], the Brazil nut allergen Ber e 1 [54] and the sesame allergen Ses i 1 [231]. The presence of bile salts may also affect the digestion of proteins, where Gass *et al*. [40] showed that the proteolysis with pancreatic enzymes trypsin and chymotrypsin of the cow's milk allergens BLG and BSA was greatly accelerated by addition of bile salts, while the digestion rate of the hen's egg allergen OVA and the minor soy allergen soybean trypsin inhibitor (STI) were unaffected. These studies show that addition of surfactant may influence the susceptibility to digestion, by either raising or decreasing the digestibility.

Food processing may also affect the susceptibility of allergens to digestion. For example heat treatment of proteins prior to digestion may affect the digestibility. In a study by Takagi *et al*. [215] it was shown that preheating of OVA before digestion by pepsin in SGF significantly accelerated the digestibility. While the OVA was stable without preheating, it was rapidly digested within 0.5 min following preheating. Also, heating of BLG significantly increased its susceptibility to digestion in a time and temperature dependent manner [259]. In contrast heating of OVM, Ber e 1 or Ses i 1 prior to digestion did not alter the digestion pattern [54;202;231]. Since many foods containing allergenic proteins are commonly eaten in a processed state, these studies imply the importance of assessing of digestibility of such proteins after processing.

The food matrix may also influence digestibility. This has been shown by Macierzanka *et al*. [242], who demonstrated that when digesting *β*-casein in an emulsified form the digestion rate was increased but on the other hand some peptide fragments were more resistant to digestion compared with the digestion of *β*-casein in no emulsion. In the same study it was shown that the susceptibility of BLG was increased if presented in an emulsified form. The conclusion was that emulsification led to adsorption-induced changes in either the conformation or the flexibility of the allergens, rendering them more easily digested in simulated gastro-duodenal digestion [242]. Polovic *et al*. [50] studied the matrix effect of the plant polysaccharides pectin, and showed that the polysaccharides decreased the digestibility of the kiwi allergen Act c 2. Also for BLG, addition of polysaccharides significantly reduced the digestibility in simulated gastro-duodenal digestion [55;260;261]. Polysaccharides were also shown to modify the digestibility of peanut allergens, however, the pattern of digestion was dependent of the type of polysaccharides [262]. As also suggested by Ofori-Anti *et al*. [217] these studies show that investigation of digestibility using purified proteins, not taking into account the possible effect of the food matrices, could potentially be misleading, providing result which may differ from those received with digestion of whole food extracts.

Evaluation of residual intact protein and peptide fragments formed during the digestion process has most commonly been accomplished by the use of SDS-PAGE and/or western blotting but other methods have also been used [20]. The methods used for detection of residual intact allergen and emerging peptide

fragments may greatly influence the resulting outcome and thereby the interpretation of the digestibility assay.

Several studies have shown the significant implication that the choice of detection method may have on the outcome of the assessment of resistance to digestion. For example Lucas *et al*. [201] reported that immunoreactive peptide fragments of kiwi allergens could be detected by immunoblot but not with coomassie staining. Diaz-Perales *et al*. [235] further showed that neither protein staining nor IgE immunoblotting with sera from allergic patients resulted in the detection of any peptide fragment bands in SDS-PAGE from the digestion process of the avocado allergen Prs a 1, which was shown to be a food allergen highly susceptible to digestion. However, peptides resulting from the digestion of the Prs a 1 were shown to have similar inhibitory potency as the intact Prs a 1 in both immunoblot- and ELISA-inhibitory experiments. They could also induce positive SPT responses in 5 out of 8 allergic patients. The sizes of peptide fragments responsible for these reactions were 5.1, 2.5 and 1.4 kDa. Thomas *et al*. [56] even reported that the type of electrophoresis gel and fixation techniques could influence the detectability of peptide fragments [56]. Such results indicate the importance of evaluating residual intact protein and peptide fragments hereof in assays with appropriate sensitivities, and that the use of more than one method could be worthwhile. Goodman and Hefle [218] pointed to the importance of choosing suitable detection methods, since proteins may have different staining ability because of different amino acid composition. For these reasons Ofori-Anti *et al*. [217] suggested the inclusion of a 10% diluted control sample of undigested protein in the assay gel to control for differences in staining between proteins and between experiments. Since evaluation of SDS-PAGE fractionation of peptide fragments resulting from the digestion process may in some situations only allows for detection of fully stable allergens and/or permanent peptide fragments of adequate large size [235], Vieths *et al*. [53] implicated the need of using immunological assays to investigate the potential allergenicity of food proteins instead of monitoring the degradation of bands by analytical electrophoresis. In a study by Mandalari *et al*. [42], residual intact BLG after simulated gastro-duodenal digestion was evaluated in SDS-PAGE as well as with RP-HPLC. While visualisation of bands in SDS-PAGE indicated that no intact BLG was left after the digestion process, RP-HPLC showed that around 10% residual BLG was left intact. Similar results were obtained for the kiwi allergen Act d 2 [239]. These studies imply that evaluation of residual intact protein with other protein-chemical methods in addition to SDS-PAGE may be very useful.

Since several factors may influence the outcome of the digestibility assay, and only small variations in the assay conditions may significantly influence the outcome, standardisation of the assay conditions would definitely make sense as emphasised several times [56;120;217]. As the evaluation of allergenic risk potential is considered for human intake, it would in addition make sense to use *in vitro* models, mimicking the average human digestion process, and thereby including in addition to simulated gastric digestion a simulated duodenal digestion process.

The variability in *in vitro* digestion results as a result of different experimental conditions suggests that variations in digestion affect the peptide profile presentation to the intestinal mucosal immune system and hence the potential allergenicity of dietary proteins. This was indicated in the study by Dupont *et al*. [39] who showed that changes in the pH as well as addition of PC could greatly influences the residual allergenicity of the gastric as well as gastro-duodenal digests. Heating prior to digestion decreased the eliciting capacity of BLG [259], and the presence of polysaccharides influenced the allergenicity of digestion products of peanut allergens, with the IgE reactivity being decreased in a degree dependent on the type of polysaccharides present [262].

No perfect digestion method exists or will ever exist, but it seems reasonable to carefully consider under which condition proteins should be digested, depending on propose of the study.

Assessment of the allergenic potential of digestion products

As discussed in the previous sections the relationship between resistance to digestion and allergenicity is not straightforward. Therefore digestibility assays should be combined with immunological assays. This would allow for an elucidation of the role of peptide fragments emerging from the digestion process, by an assessment of their allergenic capacity. The following questions could be addressed by conducting immunological assays in connection to the evaluation of susceptibility to digestion; do the peptide fragments generated during the digestion process retain IgE binding and eliciting capacity equivalent to the parent allergen? Will the IgE binding and eliciting capacity be reduced or even abolished, or could the peptide fragments be even more allergenic than the parent allergen? In addition implementation of animal studies could answer questions like; do the peptide fragments generated during the digestion process retain sensitising capacity? Will they simply be ignored by the immune system or will the peptide fragments be able to induce tolerance mechanisms? Based on knowledge from vaccine research with mixtures of small peptides, all three situations may occur, having shown peptides to be ignored, induce tolerance or induce active immunity [263;264].

In food allergy, the sizes of absorbed peptides and thereby the sizes of peptides encountered by the cells of the immune system could theoretically be of significant importance at several levels. For example, Moldaver and Larché [265] outlined that intact allergens but not small peptides corresponding to sequences of the primary structure of the allergen, may activate APCs by a cross-linking of allergen-specific antibodies bound to Fc receptors on the surface of the APCs (Figure 7A). Activated APCs may subsequently present processed peptides representing potential T cell epitopes to allergen-specific T cells for T cell activation in the presence of appropriate co-stimulatory signals [61;266]. Contrary, small peptide fragments may not be big enough to cross-link the specific antibodies on the APCs, subsequently leading to quiescent APCs, where peptide presentation to T cells results in the induction of immune tolerance [265]. Likewise, cross-linking of surface expressed antibodies have been demonstrated to be of major importance for the activation of naïve B cells [267] as well as memory B cells [268] (Figure 7B). Studies have shown that cross-linking of surface expressed antibodies on B cells by multivalent molecules initiate signalling cascades that targets the molecule for processing and subsequently presentation to specific T cells (see section 1.1.3) and eventually lead to activation of naïve B cells in the presence of appropriate co-stimulatory signals [269;270]. In contrast, univalent molecules have been shown to be significantly less efficient in such activation processes [271]. In another study it was shown that only a dimeric and not a monomeric allergen could activate memory B cells for specific IgE secretion in a secondary immune response [268]. Further, it is well recognised that cross-linking of FcεRI bound IgE antibodies is a requisite for the degranulation response of mast cells and basophils (Figure 7C) [11;12;125].
Nevertheless, there is no evidence for a specific MW or amino acid sequence length above which such cross-linking may be possible if two epitopes exist within close vicinity. The general opinion though appears to be that cross-linking acquire a molecular size of a minimum of 3.5 kDa [14;19;212;213], corresponding to around 30 amino acids.

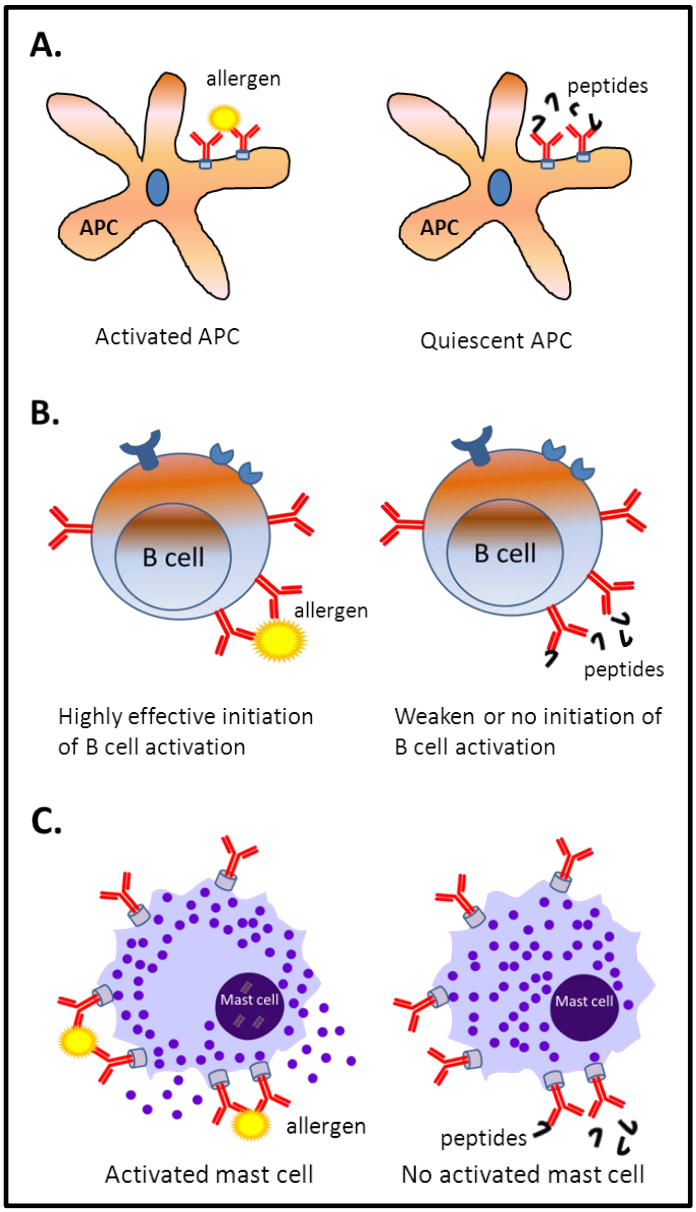

Figure 7. Cellular levels where the sizes of peptides theoretically could be of great importance for the development of an allergic response. Cross-linking events may have significant impact on the activation of; **A.** Antigen presenting cells (APC), **B.** B cells and **C.** mast cells and basophils. **A.** Cross-linking of allergen-specific antibodies bound to Fc receptors on the surface of APC may lead to activation of the APC, subsequently resulting in activation of T cells, whereas failure of cross-linking may lead to a quiescent APC, subsequently resulting in induction of regulatory T cells. **B.** Cross-linking of surface expressed antibodies on a naïve B cell may lead to activation of T cells and subsequently activation of the naïve B cell, whereas failure of cross-linking may lead to a significant weakening of such activation steps. Cross-linking of surface expressed antibodies on a memory B cell may lead to activation and differentiation into an IgE secreting plasma cell, whereas failure of cross-linking will not result in such activation and differentiation events. **C.** Cross-linking of FcεRI-bound IgE antibodies on a mast cell or a basophil may lead to degranulation response and subsequently an allergic reaction, whereas failure of cross-linking will not lead to any mediator release response.

To evaluate the effect of digestion on the allergenicity of food allergens, three distinct molecular properties need to be addressed; (1) the ability to bind IgE antibodies, (2) the ability to elicit an allergic reaction, as well as (3) the ability to sensitise, as these are the features of 'complete' allergens [115].

Several studies have evaluated the IgE binding capacity of digests generated during the *in vitro* proteolysis, showing that different outcomes may be achieved. A study on the effect of peptic digestion on the IgE binding ability of the peanut allergen Ara h 3 and the soy allergen glycinin showed that these proteins did not retain any IgE binding capacity after 120 min of digestion [224]. Likewise a study by Untermayr *et al.* [210] showed that 5 sec of peptic digestion were sufficient to abrogate the IgE binding capacity of caviar proteins. Other studies found that the IgE binding capacity was retained after digestion, though in a reduced manner. For example, Takagi *et al.* [202] found that one fifth of OVM allergic patients had serum IgE that could bind to the pepsin digested OVM which contained peptide fragments with sizes between 4.5 and 6.0 kDa. Such result was confirmed by studies of Urisu *et al.* [203] and Yamada *et al.* [219] who also found pepsin digested OVM to bind IgE, though at a reduced level compared to intact OVM. Likewise, the IgE binding capacity of cod allergens were retained after pepsin digestion though in a strongly reduced degree compared to the parent protein [57]. Again, other studies have shown that the IgE binding capacity

of digestion products equal the IgE binding capacity of the intact allergen. For example, gastro-duodenal digestion products of the grape allergen Vit v 1 were found to have IgE binding capacity similar to that of the intact Vit v 1 [43] and peptic digestion products of the cow's milk allergen BLG had similar IgE binding reactivity as the intact BLG [194]. At last few studies have shown the digestion products of food allergens to be even more IgE reactive than their parent allergen. This has been shown for the milk allergen BLG [193] as well as for kiwi allergens [201], and was suggested to be a result of the emerging of new epitopes, not accessible prior to digestion.

Only a minority of the studies, which have evaluated the IgE binding capacity of digestion products, have made the effort to correlate the binding capacity to the sizes of the peptide fragments generated during the digestion products. However, epitope mapping studies have contributed to the knowledge of IgE binding capacity of small peptides, collectively demonstrating that even very small peptides representing short amino acid sequences of the primary structure of food allergens may indeed contain the ability to bind IgE [172;196;197;272].

Studies have demonstrated that caution should be taken when evaluating the IgE binding capacity of peptide fragments and that the immunologically assay should be chosen with care, because of the great impact it may have one the resulting outcome. For example, Diaz-Perales *et al*. [235] showed that immunoblotting with sera from avocado allergic patients did not reveal any IgE binding of digestion products from the avocado allergen Prs a 1, a dietary protein highly susceptible to peptic digestion. However, the digestion products were shown to have an inhibitory potency equally to that of the intact allergen in both immunblot- and ELISA-inhibitory assays. Similar results were found in another study, revealing that while digestion products of hazelnut allergens showed no IgE binding capacity when tested by immunoblotting, the digestion products had a very strong binding capacity in EAST assays [53].

Not only IgE binding but also the association between stability to digestion and allergic eliciting capacity has been studied. When studying elicitation the biological relevance of the IgE-peptide interaction is studied.

Similarly to the above described IgE binding results, studies of elicitation capacity of digestion products have likewise shown different allergens to give different results, showing digestion products to either lose the eliciting capacity [57], retain the eliciting capacity though in a reduced manner compared to the parent protein [235;259] or retain the eliciting capacity with a magnitude equal to that of the parent protein [43;222]. For example, Eiwegger *et al.* [222] showed that the gastric as well as gastro-duodenal digestion products of the peanut allergen Ara h 1, containing peptide fragments of sizes less than 6 kDa had a HR pattern from basophils identical to that of the intact Ara h 1. Few studies correlated elicitation results to the sizes of the peptide fragments.

A study by Untermayr *et al*. [57], showed that digestion products of cod allergens retained IgE binding capacity but not eliciting capacity, demonstrates that an IgE binding capacity do not necessary correlate with a capacity to trigger allergic reactions and that additional requirement are set for peptides to inhere eliciting capacity compared to IgE binding capacity.

For ethical reasons sensitisation studies cannot be performed in humans. Animal models have been regarded as a good alternative [273;274]. Although an evaluation of sensitising capacity has only been performed with a limited numbers of dietary proteins, several murine models have been regarded as good candidates for animal models of food allergy. This is among other things based on their ability to discriminate between allergenic and non-allergenic proteins as well as discriminating between allergens of varying allergenicity [275;276]. The ability to mount a specific IgE response against dietary allergens must be the first requirement for a good animal model of food allergy. This ability has been reported to be seen

in the C3H/HeJ mouse strain [276], the Balb/c mouse strain [247;277;278] as well as in the Brown Norway rat strain [279;280]. These murine sensitisation studies were all performed by oral route with or without use of adjuvant or by intraperitoneally (i.p.) immunisation without use of adjuvant. These studies were reported to succeed in discriminating between allergenic and non-allergenic dietary proteins and might support the implementation of validated animal models in the assessment of allergenic potential of novel proteins, as expressed in the WHO/FAO rapport from 2001 [19], The Codex Alimentarius from 2003 [21] and the rapport of EFSA from 2010 [20].
Several sensitisation studies have been performed with extracts from allergenic foods or with purified intact food allergens whilst only a few sensitisation studies have been performed with break down products originating from allergenic proteins. Yet, Aldemir *et al*. [275] and Bowman and Selgrade [281] reported that a good correlation exists between digestive lability of foods and failure to induce an IgE response via the oral route. These conclusions seem to be based on interpretation of results showing that IgE production in mice could be stimulated by oral exposure to peanut and Brazil nut, and to some extent to hen's egg white, which are confirmed allergenic foods. Oral exposure to turkey extract, a food not considered to be allergenic did on the other hand not induce specific IgE. The authors interpreted these results in connection with *in vitro* pepsin digestibility assay showing that peanut, Brazil nut and hen's egg white all contained digestion stable proteins or peptide fragments, while turkey was completely digested after 15 min [276].

The small numbers of studies which have addressed residual sensitising capacity of degradation products from food allergens, by measure of specific IgE have all been performed on milk proteins. This is probably a result of the great interest in the design of safe hypoallergenic infant milk formulas as well as the requirement for an evaluation of residual sensitising capacity of such hypoallergenic formulas [282;283]. Historically, assessment of the residual sensitising capacity have been performed by oral sensitisation studies in guinea pigs [273;284;285], however the reaginic antibody response assessed in these animals is of the IgG1a subtype, making this animal model less than perfect for studying IgE mediated allergic disease.
A sensitisation study with Sprague-Dawley rats immunised i.p. with cow's milk infant formulas based on either intact proteins, partially hydrolysed proteins (pHF) or extensively hydrolysed proteins (eHF) with the use of $Al(OH)_3$ as adjuvant, showed that the pHF as well as eHF were able to induce specific IgE, though to a degree which were 100 and 10,000 times lower, respectively, than the level induced by the formula based on intact proteins [286]. In another study, Balb/c mice were immunised i.p. with different pHFs and eHFs with $Al(OH)_3$ as adjuvant [287]. These studies showed that all pHFs were able to induce specific IgE antibodies, shown by a positive skin test after intradermal injection with intact BLG or the formula itself, while only one out of two eHFs were able to induce specific IgE antibodies. These hydrolysates could all induce specific IgG1 antibodies, though in various amounts, signifying the different degree of immunogenic potency [287]. Recently, sensitisation studies have been conducted in a mouse model of orally induced cow's milk allergy [288], where C3H/HeOuJ mice were orally sensitised with whey proteins or partially hydrolysed products hereof with cholera toxin as an adjuvant [289;290]. These studies showed that while intact whey protein could induce a specific IgE response, no detectable specific IgE antibodies were measured in sera from mice dosed with partially hydrolysed whey products. However, in one study an acute skin response was evident after whey skin challenge in the ear, suggesting some residual sensitising capacity of the partially hydrolysed whey not evident by ELISA analysis of the specific IgE [290]. In own studies it was not possible to demonstrate sensitising capacity of a protein source for eHF containing peptides up to 2.5 kDa when administered to Brown Norway rats by i.p. immunisation with or without $Al(OH)_3$ as adjuvant (own unpublished results). Animal studies have also been performed with degradation products from the milk allergen BSA [291-293]. A single i.p. immunisation of BDF_1 mice with digestion

products of BSA using $Al(OH)_3$ as adjuvant failed to sensitise the mice, in contrast to intact BSA. The studied degradation products were pepsin digested for more than 12 min. However, by two i.p. immunisation of the mice, only BSA digestion products digested for more than 40 min failed to induce IgE [292]. The MW of digestion products emerging from the digestion after 40 min was in the range of 8 to 16 kDa.

While it is regarded as mandatory for safety reasons to evaluate the residual sensitising capacity of infant formulas market as hypoallergenic [282;283], it may also be important to evaluate the potential positive immune modulatory capacity. This can be achieved by examining the products ability to induce oral tolerance to the parent dietary proteins. Oral tolerance is well recognised as the common immune response to orally administered dietary proteins [61;274] and has been demonstrated in several studies using animal models of oral administration of a food, food ingredient or a purified protein. A subsequent i.p., subcutaneous or intravenous (i.v.) immunisation step (with the same or another relevant food or protein) demonstrated suppression of specific IgE response if oral tolerance was induced compared to animals only receiving post-immunisation [273;292;294-298].

In studies examining tolerance induction by hydrolysed milk products, it was found that pHFs were able to induce oral tolerance, whereas eHFs were not [294-297]. For example, van Esch *et al*. [295] showed in CH3-HeOuJ mice that whey based pHF given by gavage could induce tolerance to whey in contrast to whey based eHF, and Fritsché *et al.* [294] showed in Sprague-Dawley rats that oral administration of whey based pHF was able to induce specific oral tolerance to BLG, whereas an eHF whey based formula was not. Similarly, Peng *et al*. [297] showed that while a pHF whey product could induce oral tolerance in naive CH3-HeN mice to BLG, BSA and ALA, eHFs could not. Neither intact pHF nor eHF could on the other hand induce tolerance in mice already sensitised to BLG, BSA and ALA. Strikingly, the same group found the same pHF whey product to be effective for induction of oral tolerance to casein. eHF based on either whey or caseins could induce tolerance in naïve mice, whereas none of the products could induce tolerance in already sensitised CH3-HeN mice [296]. Although no ubiquitous agreement for the definition of pHF and eHF exist [299], von Berg [300] reports that hydrolysed cow's milk formulas are divided into pHF and eHF depending on the degree of modification, where eHF generally contain more than 95% peptides with MW of less than 3 kDa and is primarily intended for secondary prevention, whereas the MW of peptides present in pHF has a higher percentage of peptides with a MW between 3 and 10 kDa and a much lower percentage of peptides less than 3 kDa and suggested for use in primary allergy prevention.

In a study by Dosa *et al*. [292] pretreatment of BDF_1 mice i.v. by digestion products of BSA prior to i.p. immunisation with intact BSA, showed that BSA digested 12-40 min suppressed the specific IgE response towards intact BSA, whereas pretreatment with digestion products digested for either a shorter or longer period had no significant effect on the suppressing mechanisms.

In addition to milk products, Fritsché [273] showed in Sprague-Dawley rats that moderately hydrolysed soy proteins could induce oral tolerance to intact soy proteins, whereas strongly hydrolysed soy proteins were not able to achieve this.

Together these studies show the importance of peptide composition of products used for oral tolerance induction and that the tolerogenic capacity may be inherent in peptide size combinations. That the nature of the peptides may have significant importance for the tolerogenic capacity was shown in a study by Pecquet *et al*. [298]. This study examined the ability of trypsin digested BLG and peptides hereof to induce oral tolerance to BLG by oral gavage of Balb/c mice and identified the tolerogenic peptides. They concluded that the size, sequence as well as structure of the peptides appeared to be crucial for the tolerogenic capacity of the peptides.

So far, only little is known about the correlation between peptide size and structure and sensitising capacity as well as the correlation between peptide size and structure and tolerogenic properties. Nevertheless understanding the connection, if any, between the size and structure of peptides and the sensitising versus tolerogenic capacity remains fundamental for a better understanding of the influence of digestibility of the allergenic capacity and for a better understanding of the mechanisms responsible for guidance of the immune response toward different directions.

Thesis objective

The aim of the work presented in this thesis was to study the sensitising capacity of peptides from food allergens using a Brown Norway rat model of food allergy. The Brown Norway rat is a high Ig, particularly IgE, responder strain that to a certain degree resembles atopic humans in their predisposition to develop IgE mediated allergy [279]. The Brown Norway rat model is generally accepted to be a useful model for the examination of food allergy [274], since it among other things generates IgG and IgE antibodies of similar protein specificity [279;301] as well as of similar epitope specificity [302] to those produced in humans [302]. The major peanut allergen Ara h 1 and the major cow's milk allergen BLG were used as model allergens. Pepsin or trypsin and chymotrypsin were used as enzymes for simulated gastric or gastro-duodenal digestion. The effect of digestion on the allergenicity was studied by examination of:

- The sensitising potential of well-characterised gastro-duodenal digestion products of Ara h 1 in the Brown Norway rat model and comparing the sensitising potential to different doses of intact Ara h 1. Specific antibody responses were measured by ELISAs and the biological functionality of the IgE antibodies was assessed by RBL-assay (Paper 1)
- The sensitising capacity of well characterised gastric digestion products of Ara h 1 as well as fractions hereof in Brown Norway rats. Specific antibody responses were measured by ELISAs (Paper 2).
- The IgE binding epitopes of gastro-duodenal digests of Ara h 1 and intact Ara h 1. This was achieved with sera from the sensitisation study described in Paper 1, as well as with sera from peanut allergic patients using a random phage-displayed peptide library followed by mapping of the selected IgE binding epitope mimics on the Ara h 1 surface (Paper 3)
- The sensitising capacity of well characterised gastro-duodenal digestion products of BLG as well as fractions hereof and comparing it to the sensitising capacity of intact BLG. Specific antibody responses were measured by ELISAs (Paper 4).

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PAPER 1

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ORIGINAL ARTICLE **Allergens**

Digested Ara h 1 has sensitizing capacity in Brown Norway rats

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Summary

Background Food allergies are a public health issue of growing concern, with peanuts in particular being associated with severe reactions. The peanut allergen, Ara h 1, belongs to the cupin plant food allergen family, which, unlike other structural families, appears to be broken down rapidly following gastrointestinal digestion.

Objective Using Ara h 1 as a model allergen, the ability of digested protein to sensitize has been investigated.

Methods Ara h 1 was purified from whole roasted peanuts. Intact Ara h 1 was digested in an *in vitro* model, simulating the human gastrointestinal digestion process. Digestion products were analysed for peptide sizes and their ability to aggregate. Brown Norway (BN) rats, used as an animal model, were immunized with purified intact Ara h 1 or the gastrointestinal digestion products thereof. The sensitizing capacity was evaluated by analyses of specific antibody (IgG1, IgG2a and IgE) responses and ability to trigger mediator release of rat basophilic leukaemia (RBL)-2H3 cells.

Results The present study showed that Ara h 1 was broken down, resulting in peptide fragments of sizes < 2.0 kDa, of which approximately 50% was in aggregated complexes of M_r up to 20 kDa. Ara h 1 digesta were shown to have sensitizing capacity in BN rats, being capable of inducing specific IgG and IgE antibodies. The IgE response was functional, having the capacity to induce specific degranulation of RBL cells.

Conclusion From this study, it can be concluded that lability of a food allergen to gastrointestinal digestion does not necessarily abrogate its allergenic sensitizing potential.

Keywords animal model, Ara h 1, Brown Norway rats, digestion, food allergy, peanut, peptides, sensitization

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Introduction

Food allergies are responsible for a variety of reactions including fatal anaphylaxis [1, 2]. Peanut allergy is one of the most common and serious immediate-type hypersensitivity reactions to food in terms of persistence and severity [3–5]. Unlike most other food allergies, reactions to peanut are rarely outgrown [6]. While estimates of prevalence of food allergy in general are imprecise and vary between countries and cultures [7, 8], it seems that the overall prevalence of peanut allergy is around 0.6% of the US [4] and 0.5% of the British population [5]. Peanut allergy appears to be a growing phenomenon, since there has been an increase in the prevalence over the last 10–15 years [9, 10].

The causative agents of food allergies, known as allergens, are almost entirely proteinaceous in nature, the majority of those from plant-based foods belonging to just four structural types, including the so-called cupin superfamily [11]. The peanut allergen, Ara h 1, is representative of the cupin superfamily of allergens, being a vicilin-type seed storage protein [12, 13], consisting of glycosylated subunits of molecular weight M_r 63 500 [14], which assembles into homotrimers maintained by hydrophobic interactions between amino acids at monomer–monomer contact points, the same structural regions where the majority of IgE epitopes appear to cluster [15, 16]. An abundant protein, comprising around 12–16% of peanut proteins [17], Ara h 1 is recognized by serum IgE

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from more than 80% of peanut-allergic patients, indicating that it is a major allergen [14, 18]. Epitope mapping of Ara h 1 using overlapping peptides has identified 23 independent binding sites evenly distributed along the entire linear sequence of the molecule [18].

What makes only a few protein families, including the cupin superfamily [11], so prominently as allergen families? One hypothesis is that for a protein to sensitize an individual and elicit an allergic reaction, it must survive the acidic and proteolytic environment of the gastrointestinal tract and interact with the intestinal mucosa where sensitization may occur [6, 19–21]. In a study comparing the *in vitro* stability of food allergens and non-allergenic proteins to simulated gastric fluid, it was concluded that there was an association between resistance to digestion and allergenic potential [22]. This has led to pepsin resistance being used as a predictive parameter in the allergen risk assessment of novel proteins introduced into genetically modified organisms and novel foods [23]. However, in recent years, the relationship between resistance to digestion and the allergenic potential of a protein and the validity of taking this parameter into account in the risk assessment of allergenic potential have been questioned [24, 25]. While the premise that resistance to digestion correlates with the allergenicity of a protein may still hold true for some allergens [26], this does not appear to hold true for Ara h 1, where several studies have shown that Ara h 1 is degraded relatively quickly by proteases, although several peptide fragments persisted [15, 27–29].

Ara h 1 is labile to digestion and is a major allergen [14, 24]. Low-molecular-weight products of digested Ara h 1 retain T-stimulatory and IgE-binding properties [29]. But does digested Ara h 1 have the ability to sensitize?

To our knowledge, the sensitizing capacity of a digested allergen has not been investigated before, so the current study was designed to investigate the effects of simulated gastrointestinal digestion on the sensitizing potential of the model cupin allergen Ara h 1, using a Brown Norway (BN) rat model of sensitization. The BN rat is a high Ig (particular IgE) responder strain, which, to a certain degree, resembles atopic humans in their genetic predisposition to react more readily with an IgE production towards allergens [30, 31]. Although no ideal animal model mimicking the major aspects of human food allergy has been developed as yet, studies have shown that food allergy models using BN rats have the potential to categorize food proteins as non-allergens and weak and strong allergens [32, 33]. Nevertheless, additional studies are still needed before BN rats can be consolidated as a useful animal model for examination of the mechanisms of food allergy and assessment of the allergenic potential of food proteins.

This study demonstrated that even though Ara h 1 was extensively degraded to peptide fragments of <2.0 kDa, these peptides still retain sensitizing potential, inducing specific antibody responses in the BN rats and mediator release of basophils. Such low-molecular-weight peptides do not normally possess the ability to stimulate humoral immune responses. Stimulation may result from the presence of a mixture of non-covalently linked peptides retaining both B and T cell epitopes or from the ability of the peptides to aggregate into larger, immunologically active complexes. These results demonstrate that gastrointestinal digestion of an allergen to small peptide fragments does not necessarily lead to elimination of the sensitizing potential.

Materials and methods

Purification and simulated digestion of Ara h 1

Ara h 1 was purified from defatted peanut meal (from roasted peanuts) as described by Eiwegger et al. [29] and was adjudged 99% pure by reverse-phase (RP) high-performance liquid chromatography (HPLC). The final concentration of purified intact Ara h 1 was 2.5 mg/mL in the sample used for *in vitro* digestion and 1.74 mg/mL in the sample used for animal sensitization studies and immunochemical examinations. Gastric and gastro-duodenal digestions were performed essentially as described by Moreno et al. [34], with the following modifications, in order to provide enzyme-free digesta suitable for animal sensitization studies. Surfactants (gastric phosphatidyl choline vesicles and duodenal model bile salt mix) were omitted to avoid adverse effects in sensitization studies. Phosphatidyl choline has previously been shown not to affect the digestion of Ara h 1 (Clare Mills, personal communication). In brief, for gastric digestion, soluble porcine pepsin was substituted with porcine pepsin immobilized to agarose beads (P0609, Sigma, Saint Louis, MO, USA). Ara h 1 (2.5 mg/mL in 0.25 M NaCl) was adjusted to pH 2.5 with 1 M HCl, and the immobilized pepsin was added to yield an activity of approximately 182 U per mg of protein. The solution was placed in a shaking incubator at 37 °C for 120 min at 200 r.p.m. Immobilized pepsin was removed by centrifugation [1000 × g, at room temperature (RT) for 2 min] and the resulting supernatant was further clarified by passing through a 0.45 μm filter (Millipore, Bedford, MA, USA). The pH of this 'enzyme-free' gastric digesta was adjusted to 7 with 1 M NaOH. For duodenal digestion calcium and Bis-Tris were added to the gastric Ara h 1 digesta and the pH was adjusted to 6.5 with 1 M HCl, and a mixture of bovine trypsin (T1763, Sigma) and chymotrypsin (C9135, Sigma) immobilized to agarose beads was added to yield an activity corresponding to 34.5 U of soluble trypsin per milligram of protein and 0.44 U of soluble chymotrypsin per milligram of protein. This yielded a solution equivalent to 2.3 mg/mL intact Ara h 1, 9.2 mM CaCl and 24.7 mM Bis-Tris, which was placed in a shaking incubator at 37 °C for 15 min at 200 r.p.m. Immobilized enzymes were again removed by

centrifugation (1000×g, at RT for 2 min) and the resulting supernatant was filtrated through a 0.45 μm filter (Millipore). The resulting 'enzyme-free' gastro-duodenal digesta were aliquoted and stored at −20 °C until required for further analysis or animal sensitization studies.

High-performance liquid chromatography analyses

Digested and intact Ara h 1 samples were applied (50 μL) to a protein (C_4 Phenomenex Jupiter 300 Å pore size, 5 μm particle size, 250×4.6 mm i.d., Phenomenex Macclesfield Cheshire, UK) column coupled to a Jasco PU-1585 triple pump HPLC equipped with an AS-1559 cooled autoinjector, a CO-1560 column oven and a UV-1575 UV detector (Jasco Ltd, Great Dunmow, UK). Proteins and peptides were eluted using 0.1% (w : v) trifluoroacetic acid (TFA) in double-distilled water as solvent A and 0.085% (w : v) TFA in double-distilled water : acetonitrile (ACN) (10 : 90, v : v) as solvent B. The column was equilibrated with 1% solvent B. Initial elution (0–5 min) was performed using 1% solvent B in the isocratic mode, followed by elution with a linear gradient of increasing concentration of solvent B from 1% to 55% over 55 min. The HPLC column temperature was maintained at 25 °C and the autoinjector at 4 °C. The eluate was monitored for protein using UV absorbance at 280 nm.

Peptide mass profiling analyses by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight

The Ara h 1 digestion process was followed by SDS-PAGE analysis under reducing conditions as described by Moreno et al. [34], using the marker protein Mark 12™ Unstained Standard (Invitrogen, Carlsbad, CA, USA), comprising 12 protein bands in the range 2.5–200 kDa.

The exact peptide mass distribution was determined by separating the peptides by RP HPLC, followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). For fractionation, a sample of Ara h 1 digesta [50 μL of 0.8 mg/mL in 0.1% TFA (w : v)] was applied to a μRPC C2/c18 SC 2.1/10 column coupled to a SMART System (GE Healthcare, Hillerod, Denmark). Peptides were eluted with a linear gradient of ACN (5–40%) over 25 min in 0.1% TFA (w : v), with a flow rate of 200 μL/min. Fractions of 100 μL were collected, dried in a vacuum centrifuge and rediluted in 3 μL Milli Q water.

For mass spectrometric analysis, 1 μL of each of the rediluted fractions were loaded onto a MALDI target, followed by addition of 1 μL 2% TFA and 1 μL α-cyano-4-hydrocinnamic acid [5 μg/μL in 70% ACN (v : v), 0.1% TFA (v : v)]. Mass spectra for the individual fractions of Ara h 1 digesta were acquired on a Bruker MALDI-TOF MS (MALDI TOF/TOF, Ultraflex II, Bruker Daltonik GmbH, Bremen, Germany) equipped with pulsed ion extraction and a 200 Hz Smart Beam™ laser (Bruker Daltonik GmbH, Bremen, Germany). All mass spectra were initially calibrated with a tryptic digest of β-lactoglobulin.

Profiling of aggregated peptides by gel permeation chromatography

Digested Ara h 1 was analysed by gel permeation chromatography (GPC) under analytical conditions at RT on a Superdex® 75 PC 3.2/30 column coupled to a SMART System (GE Healthcare). A sample of Ara h 1 digesta (50 μL of 1 mg/mL) was applied to the column and eluted at 50 μL/min with a phosphate buffer (150 mM NaCl, 31.75 mM K_2HPO_4, NaH_2PO_4), pH 7.0. The eluate was monitored for peptides by absorbance at 220 and 280 nm. The column was calibrated for molecular weight determination by injecting a standard mixture consisting of ferritin (440 kDa; Sigma, F-4503), BSA (66 kDa; Sigma, A-2153), carbonanhydrase (29 kDa; Sigma, C-3934), cytochrom C (14 kDa; Sigma, C-2506), apotinin (6 kDa; Sigma, A-1153) and vitamin B_{12} (1.3 kDa; Sigma, V-2876).

Animals

BN rats were from the breeding colony at the National Food Institute (DTU, Denmark), weaned at 3 weeks of age and then housed in macrolon cages (two per cage) with a 12-h light : dark cycle with light from fluorescent tubes from 9:00 to 21:00 hours, at 22±1 °C and 55±5% relative humidity. Rats were observed twice a day and clinical signs were recorded. Body weight was recorded weekly.

The rats were kept on a diet free from *leguminosa* for at least three generations to avoid tolerization with other legume proteins homologous to Ara h 1. Because commercially available rat diets contain soy proteins, it was decided to produce a diet in house without *leguminosa*, egg and milk (Table 1), the sources of some of the most common food allergens studied in rodent models. The nutrient content was in accordance with rodent diet AIN-93 [35] and all ingredients were ground to a similar

Table 1. Composition of the in-house produced rodent diet used for breeding of Brown Norway rats not tolerant to Ara h 1

Ingredients	Diet (g/kg)
Maize flakes	700
Protastar*	100
Fish meal	80
Maize oil	50
Cellulose	30
Mineral mixture	28
Vitamin mixture	12

The diet is free from *leguminosa*, egg and milk and based on crushed maize flakes, potato protein and fish meal as protein sources.

*A potato protein concentrate with a low content of solanine (AgroKorn, Videsbaek, Denmark).

particle size to ensure a homogeneous mixture. Diet and acidified tap water were given *ad libitum*.

The animal experiments were carried out at the National Food Institute (DTU, Denmark) facilities under the supervision of the National Agency for Protection of Experimental Animals and the in-house committee for animal care and use.

Experimental design

To examine the sensitization capacity of intact and digested Ara h 1, groups of 8–16 BN rats were immunized intraperitoneally (i.p.) with 0 (control), 1 μg (low), 50 μg (medium) or 200 μg (high) of purified intact Ara h 1 or with 200 μg of gastro-duodenal digested Ara h 1 in 0.5 mL phosphate-buffered saline (PBS, pH 7.2; 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4) per rat on days 0, 14 and 28. One week after the last immunization, the study was terminated (day 35) and blood was collected at sacrifice. I.p. models are not ideal for assessment of the allergenic potential of food proteins. However, this model was chosen because only very low amounts of purified protein are required compared with oral models. Furthermore, we wanted to examine how *in vitro* digestion affected the immunogenic and allergenic potential of Ara h 1. Using an i.p. model, *in vivo* digestion was avoided, to know what was presented to the immune system of the rats.

Positive control sera were produced by i.p. immunization of BN rats with 100 μg/0.5 mL per rat of purified Ara h 1 absorbed on 12 mg $Al(OH)_3$ at day 1 and with 10 μg/0.5 mL per rat at days 21, 35 and 49. Blood was collected at sacrifice (day 56). A pool of these sera was used for development and optimization of ELISAs and included on each plate as a positive control.

In all experiments, sera were obtained from blood samples and stored at −20 °C until analysis.

Enzyme-linked immunosorbent assays for detection of specific immunoglobulin G1, G2a and E

Serum samples were analysed for intact and digested Ara h 1-specific IgG1 and IgG2a (indirect ELISAs) and intact Ara h 1-specific IgE (antibody-capture ELISA). A positive and a negative control serum pool were included on each ELISA plate to check assay reproducibility.

Indirect enzyme-linked immunosorbent assays for detection of specific immunoglobulin G1 and G2a against intact Ara h 1 or Ara h 1 digesta. Plates (96-well, microtitre, Maxisorp, Nunc, Roskilde, Denmark) were coated with 100 μL/well of 1 μg/mL native Ara h 1 or 10 μg/mL Ara h 1 digesta in carbonate buffer (pH 9.6; 15 mM Na_2CO_3, 35 mM $NaHCO_3$) and incubated overnight at 4 °C. Between each step, the plates were washed five times in PBS with 0.05% (w : v) Tween 20 (PBS-T). Plates were blocked for 1 h at 37 °C with 200 μL/well of 1% (w : v) BSA (for Ara h 1-specific IgG1 and Ara h 1 digesta-specific IgG2a) or with 200 μL/well of 1% (v : v) rat serum (for Ara h 1-specific IgG2a) in PBS-T. A blocking step was not performed for plates used for detection of Ara h 1 digesta-specific IgG1 as optimization procedures showed no effect of blocking on background absorbance values. Twofold serial dilutions of serum (starting at 1 : 8, v : v) in PBS-T, 50 μL/well, were added and incubated for 1 h at RT. For detection, 100 μL/well HRP-labelled mouse-α-rat IgG1 or HRP-labelled mouse-α-rat IgG2a (Zymed, Berlin, Germany) diluted 1 : 2000 (v : v) in PBS-T was added to each well and incubated for 1 h at RT. The reaction was visualized by adding 100 μL/well of 3,3′,5,5-tetramethylbenzidine-one substrate (Kem-En-Tec, Copenhagen, Denmark) for 10 min and stopped with 100 μL/well of 0.2 M H_2SO_4. Absorbance was measured at 450 nm with a reference wavelength of 630 nm, using a microplate reader (BioTek Instruments, Winooski, VT, USA). Serum from untreated rats was used as negative control. The antibody titres were expressed as $\log_2$ titres and defined as the interpolated dilution (three-parameter analysis) of a serum sample leading to the mean absorbance for the negative control serum +3 SD. The detection limit was calculated to an absorbance value of 0.1 for IgG1 and 0.15 for IgG2a (KC4, BioTek Instruments).

Antibody-capture enzyme-linked immunosorbent assay for detection of Ara h 1-specific immunoglobulin E. Plates (96-well, Maxisorp, Nunc) were coated with 100 μL/well of 0.5 μg/mL mouse-α-rat IgE (Oxford Biotechnology, Kidlington, UK) in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. Washing procedures were as described above. After blocking for 1 h at 37 °C with 200 μL/well of 1% (v : v) rabbit serum in PBS-T, twofold serial dilutions of serum (starting at 1 : 8, v : v) in PBS-T, 50 μL/well was added and incubated for 1 h at RT. Plates were incubated with 100 μL/well of 1 μg/mL digoxigenin (DIG)-coupled Ara h 1 (10 : 1) for 1 h at RT. For detection 100 μL/well of HRP-labelled sheep-α-DIG (Roche, Mannheim, Germany) diluted 1 : 1000 (v : v) in PBS-T was added and incubated for 1 h at RT before development for 15 min following the procedures described above. Titre values were determined as described above. The mean absorbance values for the negative control serum +3 SD varied between 0.4 and 0.7 between plates (KC4, BioTek Instruments). As a consequence of this plate variation in background absorbance values, detection limits for calculation of titre values were set for each plate individually.

Mediator release from rat basophilic leukaemia cells

Rat basophilic leukaemia (RBL)-2H3 cells (DSMZ, Braunschweig, Germany) were cultured in Eagle minimum essential medium (MEM) with Earles salt (M2279, Sigma)/RPMI 1640 (R0883, Sigma) medium (76 : 24, v : v)

supplemented with 10% (v : v) heat-inactivated horse serum (2605088, Invitrogen), 1% (v : v) penicillin/streptomycin solution (10 000 U/10 mg/mL, P4333, Sigma), 1% (v : v) amphotericin (250 μg/mL, A2942, Sigma) and 1% (v : v) L-glutamine (200 mM, G7513, Sigma) at 37 °C in a humidified atmosphere of 5% CO_2.

RBL-2H3 cells were harvested in the stationary phase, after overconfluence had been reached, and resuspended in Eagle MEM with Earles salts supplemented with 1% (v : v) penicillin/streptomycin, 1% (v : v) amphotericin and 1% (v : v) L-glutamine to a concentration of 1.5×10^6 cells/mL. Cells were plated at 1.5×10^5 cells/well in flat-bottomed cell culture microtitre plates (Nunc) and incubated overnight (37 °C in 5% CO_2) for attachment in a box with a lid and wet tissue for moisture. The attached cells were sensitized passively with 50 μL/well of undiluted rat serum for 1 h at 37 °C in 5% CO_2. After sensitization, the plates were centrifuged (300×g, 3 min) and washed two times in Tyrode's buffer with 1% (v : v) HEPES (1 M, 15630056, Invitrogen) and 3% (v : v) fish gelatine (G7765, Sigma). Subsequently, for IgE cross-linking, the RBL-2H3 cells were incubated for 1 h (37 °C in 5% CO_2) with 100 μL/well of allergen dilutions in Tyrode's buffer with 1% (v : v) HEPES. Based on optimization studies with serum pools and because of a limited serum supply, only two concentrations of native and digested Ara h 1 (10 and 100 μg/mL) were examined for individual sera. After incubation, cells were spun down by centrifugation (300×g, 3 min) to avoid detached cells in the supernatant. Thirty microlitre of the supernatant was transferred to a second microtitre plate (Nunc), and the released enzymatic activity was detected by hydrolysis of the substrate *p*-nitro-phenyl-*N*-acetyl β-D-glucosamimide (50 μL) for 1 h. For termination of the reaction, 100 μL/well of 0.2 M glycine (NaOH) solution (pH 10.7) was added. β-hexosaminidase release was quantitatively measured spectrophotometrically at 405 nm [36]. The total release was quantified by treating the cells with Tyrode's buffer containing 1% Triton X-100 (X100, Sigma). As negative controls, Tyrode's buffer without allergen was added to each plate to measure spontaneous release (equivalent to the background reading) and as positive controls, cells were sensitized with positive control sera and subsequently stimulated with a crude peanut extract (a gift from Unilever, Sharnbrook, Bedford, UK). For control of IgE-mediated degranulation, serum-sensitized cells were stimulated with mouse-α-rat IgE (BD Pharmingen, San Diego, CA, USA) or mouse-α-rat IgG2a (Zymed, San Francisco, CA, USA). Results are expressed as the percentage of the total release after subtraction of the spontaneous release.

Statistical analysis

The ELISA results for antibody titres for the various experimental groups were compared using a non-parametric statistical analysis because normality distribution could not be obtained for all experimental groups even with various transformation procedures. The Kruskal–Wallis test was used, followed by the Wilcoxon two-sample test. Differences between the experimental groups were regarded as significant when $P\leqslant0.05$. Asterisks indicate a statistically significant difference of a given group compared with the control group. Asterisks over a horizontal line indicate a statistically significant difference between the two given groups: $^{*}P\leqslant0.05$, $^{**}P\leqslant0.01$ and $^{***}P\leqslant0.001$. Statistical analysis was conducted using the statistical analysis system (SAS, Enterprise Guide 3, Cary, NC, USA).

Results

Ara h 1 is susceptible to gastro-duodenal digestion with immobilized enzymes and is easily broken down

Ara h 1 digesta from simulated gastrointestinal digestion with soluble enzymes have been described previously [29]. In order to prevent immune responses from developing towards the digestive enzymes (pepsin, trypsin, chymotrypsin) and the protease inhibitor used to prepare these digesta, simulated gastro-duodenal digestion was undertaken with immobilized enzymes, which were removed once digestion was completed. Analysis of 'enzyme-free' digesta by RP HPLC showed that no intact Ara h 1 survived the digestion process (Fig. 1). This is indicated by the lack of a polypeptide peak running at 46–48 min, which corresponds to the intact Ara h 1 (Fig. 1, inset). Thus, intact Ara h 1 was not detected in digesta down to a level $<0.00025\%$, the estimated detection limit of the HPLC analysis.

More detailed information on the peptide mass profile of Ara h 1 digesta was obtained by analysing individual fractions, obtained with RP HPLC (graph not shown), by mass spectrometry. Figure 2 shows a histogram of the

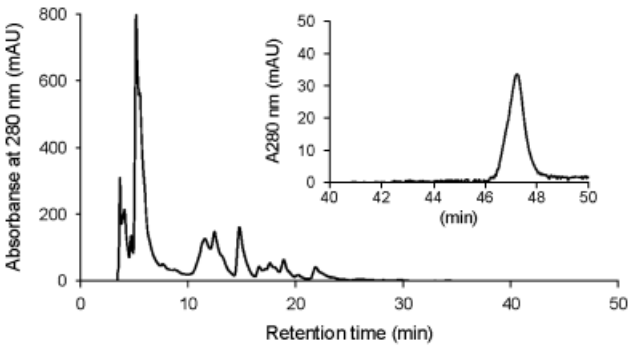

Fig. 1. Reverse-phase high-performance liquid chromatography of intact and digested Ara h 1. Comparison of the time span of elution of digested Ara h 1 made with immobilized enzymes and intact Ara h 1 (inset). For digested Ara h 1, the retention time is shown from 0 to 50 min, while the intact Ara h 1 retention time is only shown between 40 and 50 min, the time interval in which the single peak corresponding to eluted Ara h 1 appears.

peptide mass distribution of the Ara h 1 digesta. Detailed analysis showed that Ara h 1 was broken down to very small peptide fragments, which, in general, were lower than 2 kDa. More than 85% of the detected peptide fragments had masses between 0.5 and 1.5 kDa, while the rest of the peptide fragments had masses below 0.5 kDa or between 1.5 and 2.0 kDa.

Ara h 1 digesta aggregate to complexes of larger sizes

Analysis of digestion products of Ara h 1 by GPC under physiological conditions showed that, even though Ara h 1 was broken down to very small peptide fragments, these peptides occurred in complexes of larger sizes (Fig. 3). From the area under the curve, showing an absorbance at 220 nm, it appears that more than half (55.8%) of the peptides were in aggregated complexes of sizes between 2 and 20 kDa.

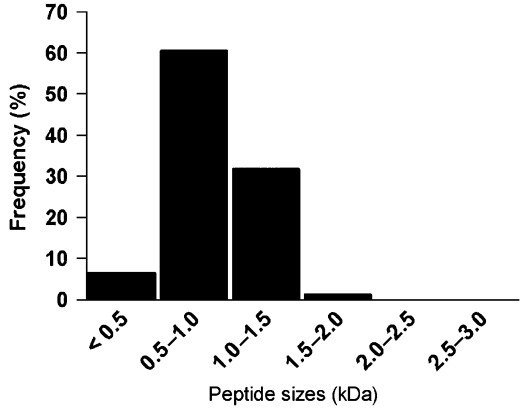

Fig. 2. Peptide mass distribution. Mass spectra of the Ara h 1 digesta made with immobilized enzymes shown in the histogram of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry-derived peptide masses, where each bar corresponds to peptide size intervals of 0.5 kDa. The figure shows the peptide mass distribution when both the number and the intensity of each peak are included.

Ara h 1 digesta are able to induce both a specific immunoglobulin G and a specific immunoglobulin E response

To examine the effect of simulated gastro-duodenal digestion on the sensitizing potential of the model allergen Ara h 1, a dose–response experiment was performed. BN rats, used as an animal model, were immunized i.p. either with 1 (low), 50 (medium) or 200 μg (high) of intact Ara h 1 or with 200 μg of digested Ara h 1. Rat sera were evaluated for specific IgG1 and IgG2a responses against both intact and digested Ara h 1 and a specific IgE response against intact Ara h 1.

The immunogenic response of individual rats within each immunization group showed that the rats responded in a dose-dependent manner towards the intact Ara h 1 (Fig. 4). While immunizations with 50 or 200 μg of intact Ara h 1 induced a statistically significant different IgG1 ($P \leqslant 0.001$) and IgG2a (50 μg: $P \leqslant 0.01$; 200 μg: $P \leqslant 0.001$) response against intact Ara h 1 compared with the control group (immunized with PBS), immunizations with 1 μg of intact Ara h 1 were not able to mount a specific IgG response different from the response found for the control group (Figs 4a and b). Interestingly, digested Ara h 1 was found to induce a significant IgG response against intact Ara h 1 compared with the control group (IgG1: $P \leqslant 0.01$; IgG2a: $P \leqslant 0.05$). Immunizations with 200 μg of digested Ara h 1 induced an almost identical IgG response against both intact and digested Ara h 1 whereas immunizations with 50 or 200 μg of intact Ara h 1 induced a significantly higher IgG response against intact Ara h 1 compared with digested Ara h 1 (50 μg: $P \leqslant 0.01$; 200 μg: $P \leqslant 0.001$). In general, both high- and low-titre sera raised against digested Ara h 1 reacted equally well with digested and intact Ara h 1. In contrast, analysis of sera from rats immunized with intact Ara h 1 showed that some of the high-responding sera bound to intact as well as digested Ara h 1 (e.g. animal no. 53, 54, 56, 57 and 60), while others only bound to intact but not digested Ara h 1 (e.g. animal no. 49, 51 and 52).

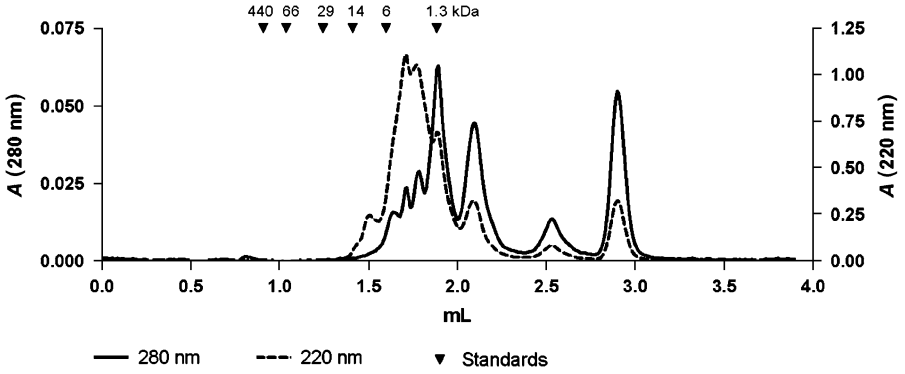

Fig. 3. Gel permeation chromatography of Ara h 1 digesta under physiological conditions. Gel permeation profile of Ara h 1 digesta made with immobilized enzymes, shown at 220 and 280 nm, respectively. Standard molecular mass markers at 220 and 280 nm are shown across the top of the graph.

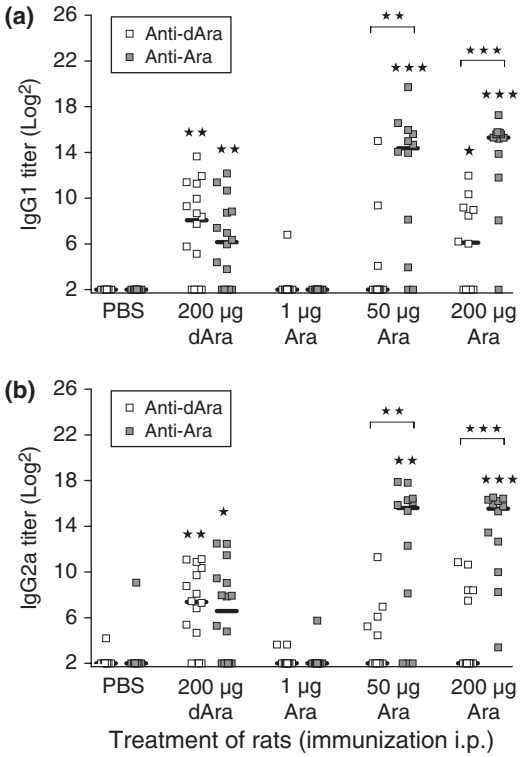

Fig. 4. Comparison of specific IgG responses induced against digested or intact Ara h 1. Groups of rats ($n = 4$–8/sex) were dosed on days 0, 14 and 28 with either phosphate-buffered saline (PBS; control), 200 µg of digested Ara h 1 (dAra) or 1 µg (low), 50 µg (medium) or 200 µg (high) of intact Ara h 1 (Ara). Serum was obtained at sacrifice (day 35) and analysed by ELISA for measurement of a specific IgG1 (a) and IgG2a (b) response against digested Ara h 1 (open symbols) or intact Ara h 1 (closed symbols). Each symbol represents an animal. Horizontal bars indicate the median values. Statistically significant difference between groups was determined using the Kruskal–Wallis test, followed by the Wilcoxon two-sample test. Asterisks indicate statistically significant differences of an allergen-dosed group compared with the control group (PBS). Asterisks over a horizontal line indicate a statistically significant difference between the IgG response against intact and digested Ara h 1 for an allergen-dosed group: $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$ and $^{***}P \leqslant 0.001$. dAra, digested Ara h 1; Ara, intact Ara h 1; Anti-dAra, specific IgG against digested Ara h 1; Anti-Ara, specific IgG against intact Ara h 1.

Figure 5 shows the IgE response towards intact Ara h 1. Also with IgE, the rats reacted in a dose-dependent manner, where a statistically significant increase in the IgE response against intact Ara h 1 compared with the control group was found for groups immunized with 50 µg ($P \leqslant 0.05$) and 200 µg ($P \leqslant 0.001$) of intact Ara h 1. In addition, IgE raised against intact Ara h 1 reacted more readily with intact Ara h 1 than IgE raised against digested Ara h 1. Immunizations with 50 or 200 µg of intact Ara h 1 induced a significantly higher IgE response against intact Ara h 1 compared with immunizations with digested Ara h 1 (50 µg: $P \leqslant 0.01$; 200 µg: $P \leqslant 0.001$). In the group immunized with digested Ara h 1, it was only possible to detect an IgE response against intact Ara h 1 for two out of 16 animals (no. 18 and 19), which were also those that had the highest IgG titres.

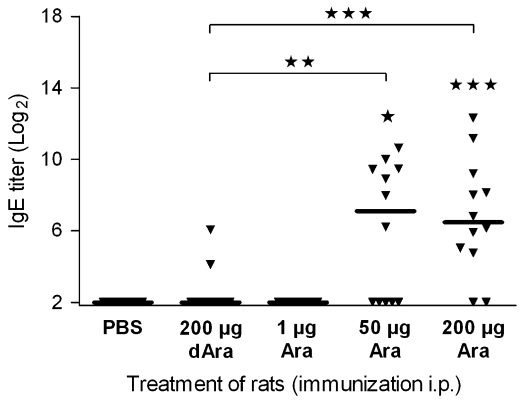

Fig. 5. Specific IgE response against intact Ara h 1. Groups of rats ($n = 4$–8/sex) were dosed on days 0, 14 and 28 with either phosphate-buffered saline (PBS; control), 200 µg of digested Ara h 1 (dAra) or 1, 50 or 200 µg of intact Ara h 1 (Ara). Serum samples obtained at sacrifice (day 35) were analysed by ELISA. Each symbol represents the specific IgE response against intact Ara h 1 for an individual animal. Horizontal bars indicate median values. Statistically significant difference between groups was determined by the Kruskal–Wallis test, followed by the Wilcoxon two-sample test. Asterisks indicate a statistically significant difference of an allergen-dosed group compared with the control group (PBS). Asterisks over a horizontal line indicate a statistically significant difference between two allergen-dosed groups: $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$ and $^{***}P \leqslant 0.001$. dAra, digested Ara h 1; Ara, intact Ara h 1.

Because IgE titres could only be determined against intact protein, given that it was not possible to couple peptide fragments to DIG, it may be that antibody responses that preferentially recognized digested Ara h 1 compared with intact Ara h 1 might be missed. Nevertheless, the antibody titre responses indicate that digestion reduced the immunoreactivity of intact Ara h 1.

Ara h 1 digesta-specific immunoglobulin E is biologically relevant

While a substance may induce an IgE response, it may not be biologically relevant, i.e. incapable of inducing histamine release and may hence trigger an allergic reaction. Consequently, the biological activity of the specific IgE responses raised in the immunized rats was tested using the RBL assay [36].

To ensure that degranulation of the RBL cells was dependent on allergen cross-linking of IgE molecules and not IgG2a molecules [37], tests were performed where sensitized RBL cells were stimulated with anti-IgE or anti-IgG2a. Anti-IgE resulted in a mediator release of approximately 88%, while anti-IgG2a resulted in no mediator release (data not shown).

A functional IgE response was obtained in rats immunized with 200 µg of intact Ara h 1 as well as 200 µg of digested Ara h 1, resulting in allergen-specific degranulation of the RBL cells (Fig. 6). These data show that within each group, there were animals with nearly maximum

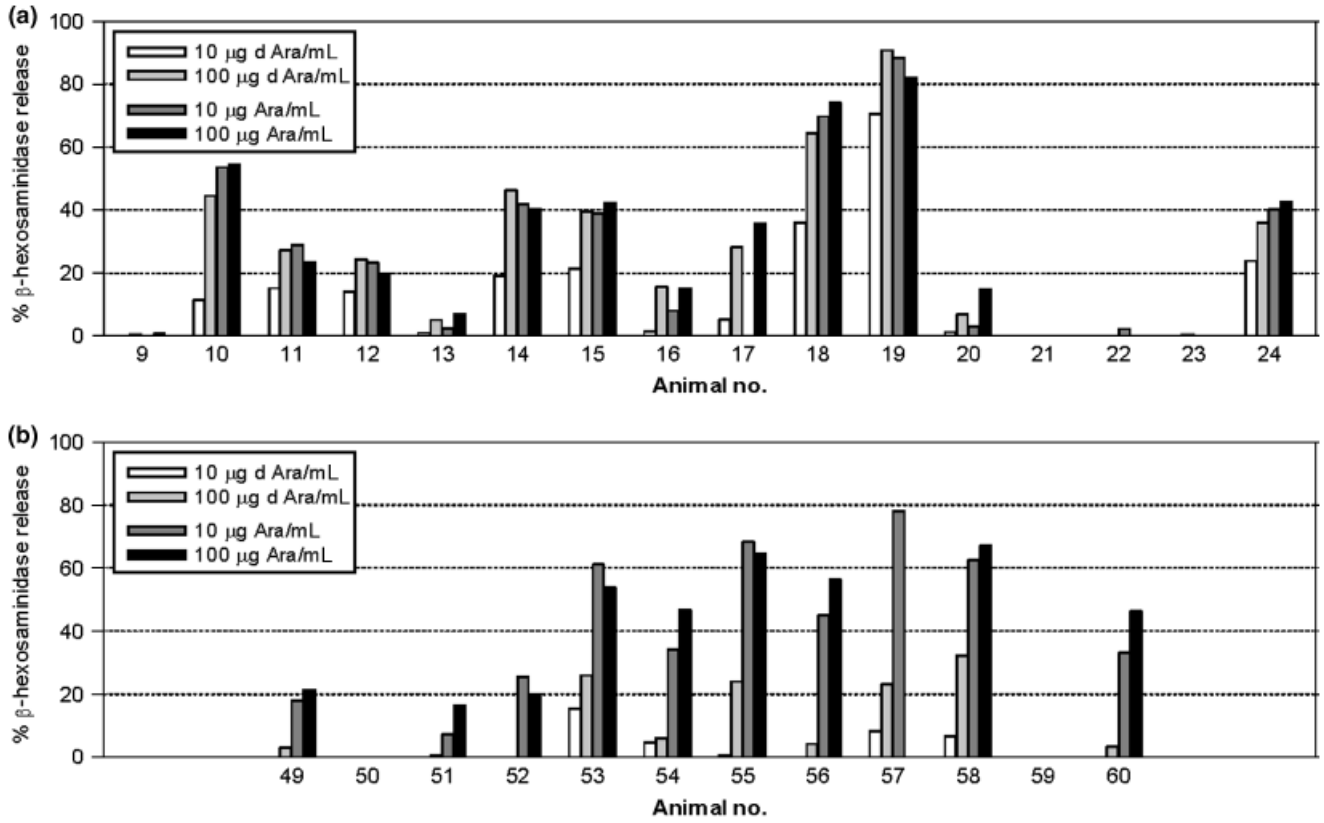

Fig. 6. Allergen-specific degranulation of rat basophilic leukaemia (RBL) cells. RBL cells were passively sensitized with undiluted serum from individual rats immunized with either 200 µg digested Ara h 1 (a) or 200 µg intact Ara h 1 (b), and subsequently stimulated with the indicated concentrations of digested Ara h 1 (10 or 100 µg/mL) or intact Ara h 1 (10 or 100 µg/mL) for degranulation. Data are presented as percentage β-hexosaminidase release of the total release. Because of a limited serum supply for rat no. 57, no result was obtained for IgE cross-linking with 100 µg Ara h 1/mL.

mediator release as well as animals with no detectable release. IgE molecules from animals immunized with digested Ara h 1 were cross-linked equally well with digested and intact Ara h 1 at the 100 µg/mL level (Fig. 6a). In contrast, IgE molecules from animals immunized with intact Ara h 1 were cross-linked much better with intact Ara h 1 compared with digested Ara h 1 (Fig. 6b). These results parallel the pattern of IgG sensitization observed by ELISA for the same animals (Fig. 4). The sensitivity of the RBL assay is much higher than the antibody-capture ELISA, and hence was able to detect IgE responses in more animals than the ELISA. Nevertheless, the only two rats (animal no. 18 and 19) for which it was possible to detect an IgE response to intact Ara h 1 by ELISA were also the ones with the highest degranulation response in the RBL assay.

Discussion

One hypothesis linking the structural attributes of certain proteins with their allergenic potential is that certain structures are more resistant to gastrointestinal digestion and hence are more effective in sensitizing via the gastrointestinal tract. This is possible based on the hypothesis that for a protein to sensitize an individual and elicit an allergic response, it must survive the acidic and proteolytic environment of the human gastrointestinal tract to be absorbed through the intestinal mucosa and sensitize via the mucosal immune system [6, 19, 21, 38]. However, most importantly, the belief is based on a study by Astwood et al. [22], who compared the *in vitro* stability of allergens and non-allergenic proteins to simulated gastric fluid and found an association between resistance to digestion and allergenicity. While this premise may hold true for some food proteins, it does not seem to hold true for the major peanut allergen Ara h 1, a member of the cupin superfamily.

The current study was designed to investigate the basis for linking gastro-duodenal digestibility and allergenicity, in terms of sensitization. We confirmed that Ara h 1 is a food protein labile to gastrointestinal digestion. When subjected to digestive conditions that simulate those found in the human gastrointestinal tract, intact Ara h 1 was broken down to peptide fragments of sizes generally <2.0 kDa. More than 50% of these peptide fragments were in aggregated complexes of sizes between 2 and 20 kDa. This was not a result of disulphide bonds sustaining some 3D structure as only one cysteine residue is left

in the Ara h 1 molecule after N-terminal cleavage [39]. Therefore, this study shows that a food protein known to be allergenic does not necessarily resist digestion. This is in agreement with studies by Fu et al. [24] and Kenna and Evens [25], who found that food allergens are not necessarily more resistant to digestion than non-allergenic proteins with a similar cellular function.

Variable results, some in agreement [27, 28] with our findings and others not [40], were obtained in previous reports on Ara h 1 digestibility. The diverse findings of the digestibility of Ara h 1 may be a result of the different enzymatic and/or chemical treatments used in the experiments. This indicates that the choice of methods used to evaluate the digestibility of an allergen is highly important for the obtained results, which is also suggested by Fu [41] and Untersmayr et al. [42].

The present sensitization study in BN rats showed that both intact and digested Ara h 1 were immunogenic, being able to induce IgG1 and IgG2a responses. Likewise, this study showed that both intact and digested Ara h 1 were allergenic, being able to induce specific and functional IgE responses.

Ara h 1-specific IgG and IgE titres in rats immunized with 200 μg digested Ara h 1 were significantly lower than Ara h 1-specific IgG and IgE titres in rats immunized with 50 or 200 μg intact Ara h 1. This indicates that the digestion process may have eliminated some epitopes. However, our results showed that some IgG and IgE antibodies raised against digested Ara h 1 could react with intact Ara h 1, suggesting that some IgG and IgE epitopes had survived the digestion process. Survival of IgE epitopes during the digestion process is most clear from the RBL results, where both intact and digested Ara h 1 were able to induce a specific degranulation response in RBL cells stimulated with sera raised against digested Ara h 1. That Ara h 1-specific IgE epitopes can survive the gastro-duodenal digestion process has also been shown in an epitope mapping study by Bøgh et al. (personal communication). Similar results were obtained by Eiwegger et al. [29], who demonstrated that Ara h 1 digesta were as potent as the intact allergen molecule in T cell activation and IgE-cross-linking experiments.

Based on the present study, it cannot be concluded whether or not neo-epitopes have developed as a result of the digestion process.

Together, these findings indicate that Ara h 1-derived breakdown products resulting from gastro-duodenal digestion still retain the potential to induce sensitization and elicitation of an allergic response. In contrast to this study, some previous studies have shown that digestion of Ara h 1 abrogates its IgE-binding capacity. Both Astwood et al. [22] and Hong et al. [43] reported that no residual IgE-binding capacity remained after gastric digestion, shown by means of immunoblotting. This may, however, be a result of the methods used to monitor the IgE reactivity. Vieths et al. [28] reported that investigation of the allergenic potential by monitoring degradation of bands by analytical electrophoresis and immunoblotting was not sufficiently sensitive. The generation of small IgE-reactive proteolytic fragments with a molecular weight lower than 10 kDa could be below the range of separation in electrophoretic systems. Instead, immunological assays should be used to assess the allergenic potential of digestive products [28]. Similar conclusions were arrived at by Diaz-Perales et al. [44].

That allergens do not necessarily have to survive the acidic and proteolytic environment of the gastro-duodenal tract to act as an allergen is a relevant issue for the risk assessment of allergenic potentials of novel proteins, because the resistance to digestion is one of the parameters in such a test regime [23]. Van Beresteijn et al. [45] reported that the minimal molecular weight to elicit immunogenicity and allergenicity of whey protein hydrolysates appeared to be between 3 and 5 kDa. Poulsen and Hau [46] showed that peptides with a molecular weight below 3.4 kDa, obtained by enzymatic hydrolysis of whey protein, were unable to sensitize mice whereas peptides with a molecular weight above 6.5 kDa were able to sensitize mice. Huby et al. [47] suggested that the minimal size for a peptide to cross-link two IgE molecules on the surface of a mast cell to initiate degranulation was at least 3 kDa. Thus, peptide fragments below the size of 3.5 kDa should not have the ability to act as a sensitizing allergen. This is not in accordance with our findings. Although Ara h 1 may have a unique epitope density such that small fragments might retain allergenicity after digestion, we suggest that the allergenic potential found in this study is primarily a result of aggregated complexes of the peptide fragments and that small peptide fragments are held together by non-covalent interactions, e.g. hydrophobic interactions. That aggregation could be an important player in sensitization potential is indicated by King et al. [48] and Hermeling et al. [49]. The allergenicity of the digestive products of Ara h 1 could also partly be a result of synergistic effects. It has been observed that there is a strong synergistic effect by co-immunizing mice with a mixture of different peptides in small and equal amounts compared with immunization with a single peptide in a much higher quantity [50–53].

In summary, digested Ara h 1 was both immunogenic and allergenic. This can only be explained if peptides within the digesta mixture comprise both B and T cell epitopes and/or the presence of peptide aggregates that are able to induce an immune response and cross-link IgE. These data also indicate that while gastrointestinal digestion may be relevant for certain structural types of allergens, including the lipid transfer proteins [26] and 2S albumin allergens, this does not necessarily hold true for other structural types of allergens such as Ara h 1 belonging to the cupin superfamily. It also demonstrates

that *in vitro* digestion using the pepsin assay approach developed by Astwood et al. [22] as part of the risk assessment of allergenic potentials of novel proteins [23] needs to be linked with other studies, such as animal sensitization.

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PAPER 2

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Digested Ara h 1 Loses Sensitizing Capacity When Separated into Fractions

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ABSTRACT: The major peanut allergen Ara h 1 is an easily digestible protein under physiological conditions. The present study revealed that pepsin digestion products of Ara h 1 retained the sensitizing potential in a Brown Norway rat model, while this sensitizing capacity was lost by separating the digest into fractions by gel permeation chromatography. Protein chemical analysis showed that the peptide composition as well as the aggregation profiles of the fractions of Ara h 1 digest differed from that of the whole pool. These results indicate that the sensitizing capacity of digested Ara h 1 is a consequence of the peptides being in an aggregated state resembling the intact molecule or that most peptides of the digests need to be present in the same solution, having a synergistic or adjuvant effect and thereby augmenting the immune response against other peptides.

KEYWORDS: *Ara h 1, digestion, animal model, food allergy, peptides, aggregation*

INTRODUCTION

Food allergy most often involves an allergen-specific IgE antibody-mediated immunologic response. It is an adverse reaction to an otherwise harmless food or food component that involves an abnormal response of the immune system to specific food proteins. One of the major unanswered questions in food allergy research is what makes a protein a food allergen. Yet, no definite answer to this exists. However, one of the hypotheses has been that for a protein to be a food allergen, it must survive the digestion process through the gastrointestinal tract, to reach the immune system as an intact protein or as large peptide fragments.[1,2] The first systematic assessment of food allergen digestibility was conducted in 1996 by Astwood et al.[3] They showed that in general food allergens were resistant to pepsin digestion, whereas nonallergenic proteins were more easily digested. Since this, several studies examining the correlation between resistance to digestion and allergenicity have been made, where the correlation between stability and allergenicity was less clear.[4−10] However, it may still be reasonable to think that proteins being resistant or at least partially resistant to digestion have an increased probability of reaching the intestinal mucosa in a form that is sufficiently immunologically active to sensitize the mucosal immune system and be sufficient in size to retain the ability to cross-link two IgE molecules and thereby elicit an allergic reaction. The stability to digestion is for those reasons also recommended for use in the safety assessment of newly introduced proteins in genetically modified foods based on a decision tree or a weight of evidence approach, which includes, among a variety of tests, the assessment of resistance to digestion by pepsin.[11−13] However, while pepsin stability as a part of an allergenicity assessment would still seem reasonable for the purpose of safety evaluation of most food proteins, we now know that for some allergenic proteins, this approach would be misleading. The milk allergen β-casein (Bos d 8)[7,14,15] as well as the peanut allergen Ara h 1[9,10] have several times been shown to be easily digestible food allergens.

Peanut allergy is one of the most common and serious types of IgE-mediated food allergies in terms of persistency and severity[16,17] and seems to be an increasing problem in the western world.[18,19] The peanut protein Ara h 1, which is a major allergen,[20,21] is a 7S globulin protein belonging to the cupin superfamily of allergens.[22] Ara h 1 is a homotrimeric protein, consisting of 63.5 kDa large subunits,[20] held together by hydrophobic interactions between amino acids at monomer−monomer contact points.[23,24] Ara h 1 is a readily digestible allergen, being digested to small peptide fragments by gastroduodenal digestion.[9,10] Even though Ara h 1 is a labile protein, Eiwegger et al.[9] and Bøgh et al.[10] showed that the digestion products of Ara h 1 retain allergenic potential, being able to sensitize as well as elicit allergic reactions. These studies indicated that aggregation of peptides may play a major role in maintaining allergenic activity. Epitope mapping studies of this protein have suggested both linear[21] and conformational[25] IgE-binding epitopes, at least some of which are able to survive the digestion process.[10,26]

Peptides need to have a certain size to be allergenic, but the exact lower molecular weight (MW) size limit is not known. Yet, many suggestions for such a lower MW size limit have been presented,[11,27−31] and the general view appears to be that peptides need to be approximately 3.5 kDa to contain sensitizing and eliciting allergenic capacity. This may be a realistic limit for some allergen-derived peptides. We have previously

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shown that peptide fragments, as small as 2 kDa, have the capacity to sensitize and elicit allergic reactions.[10] In the present study, we focus on examining under which conditions such peptide fragments retain their allergenic potential.

The objective of this study was to increase our knowledge and understanding of the allergenic capacity of small peptide fragments. This was done by using the known major peanut allergen Ara h 1 as a model allergen, based on the knowledge that this allergen retains its allergenic potential when digested to small peptide fragments while using pepsin as the enzyme for digestion. Digestion products of Ara h 1 and fractions hereof were thoroughly characterized, and examination of sensitizing capacity was performed using a Brown Norway (BN) rat model for food allergy.

MATERIALS AND METHODS

Purification of Peanut 7S Protein Ara h 1. Raw redskin peanuts (Julian Graves LTD, Kingswinford, United Kingdom) were skin peeled. Peanuts were frozen with liquid nitrogen and blended in a steel blender until a fine texture was obtained. Subsequently, the crushed peanuts were taken through two rounds of defatting [peanut:hexane, 1:5, w:v, 1 h, room temperature (RT)] and further homogenized in a coffee grinder.

Proteins were extracted in double-distilled water (peanut:water, 1:5, w:v) with 0.02% sodium azide (v:w), containing a protease inhibitor tablet (Roche complete mini protease inhibitor tablet, Roche, Sussex, United Kingdom) for 1 h at RT. After clarification by centrifugation (3 000*g*, 20 min) ammonium sulfate was added to a saturation of 70% and centrifuged (30 000*g*, 30 min). The supernatant was dialyzed against buffer (Tris 20 mM, pH 7.5, 500 mM NaCl) at 4 °C, and samples of 40 mL were applied to a column of 10 mL of Con A Sepharose (GE Healthcare, Buckinghamshire, United Kingdom). Unbound protein was removed by washing with the buffer, while pure Ara h 1 was eluted by addition of 400 mM methyl α-D-mannopyranoside. For further purity, eluted Ara h 1 was applied to a column of Superdex 200 prep grade (HiLoad 16/60 and 26/60 Superdex 200 prep grade, GE Healthcare) and eluted with 25 mM Tris, pH 7, and 150 mM NaCl. Purified Ara h 1 was filtrated through a Millipore filter paper (0.22 μm, Millipore Corp., Bedford, MA) with vacuum and afterward ultrafiltrated through an ultrafilter membrane (pore size, 10 kDa) with gas (argon, 10 psi). The Ara h 1 was dialyzed against 150 mM NaCl, and the concentration was determined by UV absorbance reading at 280 nm. Furthermore, concentration of purified Ara h 1 was determined by amino acid analysis to be 4.38 mg/mL. The purified intact Ara h 1 was analyzed for the presence of endotoxin by Lonza endotoxin testing service (Lonza, Verviers, Belgium).

N-Terminal Sequencing of Intact Ara h 1. To analyze the isotype composition of intact Ara h 1, amino terminal sequencing was performed. Protein sequencing of the intact Ara h 1 (5 μL, 16 pmol/μL) was carried out by automated N-terminal Edman degradation in a Procise 494 sequencer (Applied Biosystems, Foster City, CA) in liquid phase mode.

Simulated Gastric Digestion of Ara h 1. Gastric digestion was performed essentially as described by Bøgh et al.[10] In short, pepsin immobilized to agarose (P0609, Sigma, St. Louis, MO) was washed two times (100*g*, 1 min) in 10 mL of 1 mM HCl. Purified Ara h 1 (2.4 mg/mL in 150 mM NaCl) was adjusted to pH 2.5 with 1 M HCl and added to the immobilized pepsin to yield an activity of pepsin of approximately 170 U per mg Ara h 1. The solution was placed in a shaking incubator (200 rpm, 37 °C) for 120 min. Reaction was stopped by adjusting the pH to 7 with 1 M NaOH, centrifugation (1 000*g*, RT, 2 min), and filtration of supernatant through a 0.45 μm Millipore filter followed by a 0.22 μm filter.

Separation of Digested Ara h 1 into Fractions. For fractionation of the digested Ara h 1, preparative gel permeation chromatography (GPC) was performed. The digested Ara h 1 (6 mL, 2.3 mg/mL) was loaded onto a Superdex 75 prep grade, HiLoad 26/60 column (GE Healthcare, Uppsala, Sweden) connected to GradiFrac system (Pharmacia Gradifrac FPLC system, GE Healthcare). Peptides were eluted at RT with 150 mM NH_4HCO_3, pH 7.8, at 1 mL/min and collected in fractions of 4 mL. The eluted peptides were detected by absorbances at 280 and 226 nm. Four runs were made to fractionate all digested Ara h 1. The column was calibrated for MW determination by applying a standard mixture consisting of 1 mg/mL ferritin (440 kDa; F4503, Sigma), 0.75 mg/mL ovotransferrin (79 kDa; C-0880, Sigma), 1 mg/mL carbonic anhydrase (29 kDa; C-3934, Sigma), 1 mg/mL cytochrome C (14 kDa; C-2506, Sigma), 2 mg/mL apotinin (6 kDa; A-1153, Sigma), and 0.1 mg/mL vitamin B_{12} (1.3 kDa; V-2876, Sigma).

Fractions from the four consecutive runs were collected and pooled according to the GPC profile (Figure 1) in three different pools

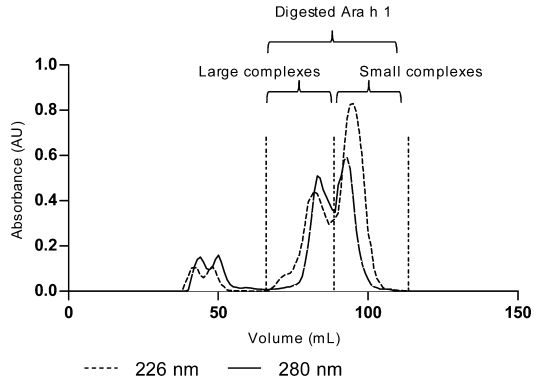

Figure 1. Preparative GPC of Ara h 1 digests. For fractionation of the Ara h 1 digest, preparative GPC was performed in 150 mM HN_4HCO_3, where Ara h 1 digest was separated into fractions based on the chromatographic profile. From the profile, shown with absorbances at 226 and 280 nm, it was decided to make three different pools, indicated by the vertical dashed lines. The pools are referred to as: digested Ara h 1, large complexes and small complexes, where digested Ara h 1 is the whole pool of digest, consisting of both the fraction of large and the fraction of small complexes. The first double peak had an apparent MW of 60−440 kDa, indicating that some intact Ara h 1 could be left in this fraction. To avoid the possible presence of any intact Ara h 1 in the digests, this double peak was excluded.

(in the following designated: digested Ara h 1, large complexes, and small complexes), where digested Ara h 1 constitute the fraction of large and small complexes. The pools were placed at −80 °C for a minimum of 1 h, afterward freeze-dried for approximately 48 h, and rediluted in Milli Q water [water drawn from a Milli Q System equipped with an Organex cartridge from Millipore (Bedford, MA)] to give a concentration of approximately 1 mg/mL.

Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) Analysis. For analysis of purity and residual intact Ara h 1 in the digests, analytical RP-HPLC was performed. Samples (40 μL, 1 mg/mL) were applied to a μRPC C2/C18 SC 2.1/10 column (120 Å pore size, 3 μm particle size, 100 mm × 2.1 mm i.d., GE Healthcare) connected to a SMART system (GE Healthcare). Chromatography was performed at RT using 0.1% trifluoroacetic acid (TFA) in Milli Q water (v:v) as solvent A and 0.1% TFA in Milli Q water:acetonitrile (ACN) (10:90, v:v) as solvent B. Elution was performed at a flow rate of 200 μL/min for 2.5 min with 5% solvent B, followed by elution with a linear gradient of increasing concentration of solvent B from 5 to 50% for 22 min. Elution profiles were monitored using UV absorbance at 220 and 280 nm. Fractions of 100 μL were collected, dried in a vacuum centrifuge, and rediluted in 3 μL of Milli Q water for analysis by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS).

Amino Acid Analysis. For examination of amino acid composition and quantification, amino acid analysis was performed according to Barkholt and Jensen[32] after hydrolysis overnight in HCl.

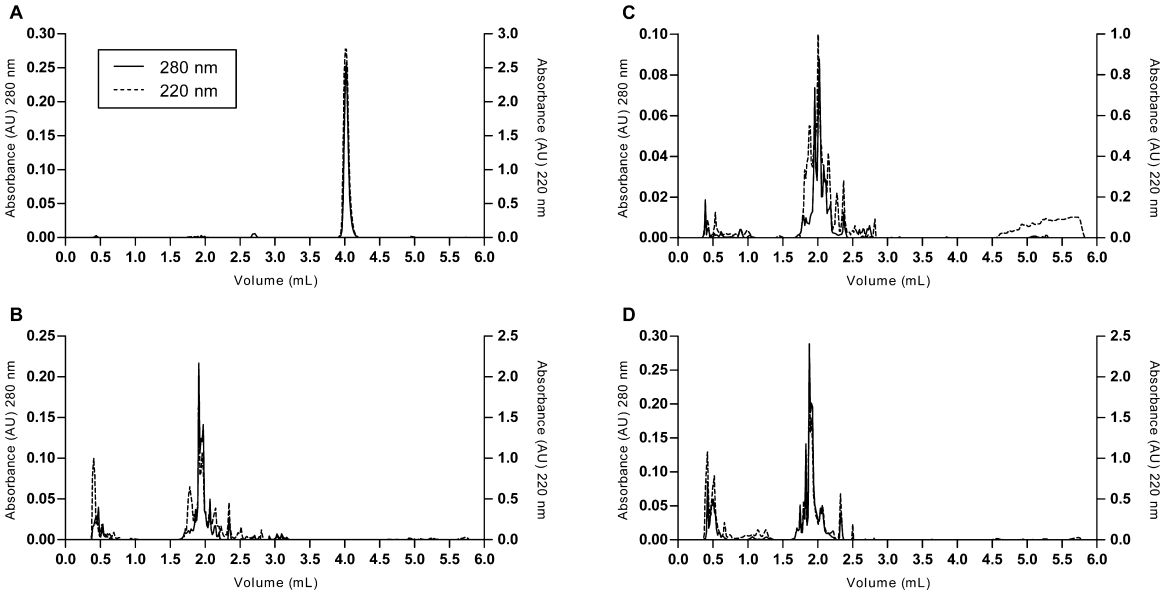

Figure 2. Analytical RP-HPLC. Comparison of chromatography profiles performed with 0.1% TFA/ACN for intact Ara h 1 (A), digested Ara h 1 (B), large complexes (C), and small complexes (D), shown with absorbances at 280 and 220 nm.

MALDI-TOF MS. For analysis of peptide mass distribution in samples of Ara h 1 digests, MALDI-TOF MS was performed on a Bruker MALDI-TOF MS (MALDI TOF/TOF, Ultraflex II, Bruker Daltonik GmbH, Bremen, Germany) equipped with pulsed ion extraction and 200 Hz Smart Beam laser. One microliter of the rediluted fractions from RP-HPLC was loaded onto a MALDI target, followed by addition of 1 μL of 2% TFA and 1 μL of α-cyano-4-hydrocinnamic acid [5 μg/μL in 70% ACN (v:v), 0.1% TFA (v:v)]. All mass spectra were initially calibrated with a tryptic digest of β-lactoglobulin.

GPC Analysis. For analysis of aggregation profiles in samples of Ara h 1 digests, analytical GPC was performed. Samples (40 μL, 1 mg/mL) were applied to a Superdex 75 PC 3.2/30 column (GE Healthcare) connected to a SMART system (GE Healthcare). Chromatography was performed at RT with a flow rate of 50 μL/min using 150 mM HN_4HCO_3 (pH 7.8) as the eluent. The eluent profiles were monitored using UV absorbances at 220 and 280 nm. The column was calibrated for MW using 12 μL of the standard mixture previously described.

Animals. BN rats were from the in-house breeding colony at the National Food Institute (DTU, Denmark), weaned at 3 weeks of age and then housed in macrolon cages (two per cage) with a 12 h light:dark cycle, at 22 ± 1 °C and 55 ± 5% relative humidity. Rats were observed twice daily, and clinical signs were recorded.

Rats were kept on diet free from leguminous fruit for three generations to avoid tolerance against Ara h 1. Rat diet was produced in-house and based on rice flour, potato protein, and fish meal as protein sources, as previously described,[10] with the exception of maize flakes being substituted with rice flour. Diet and acidified water were given ad libitum. Animal experiments were carried out at the National Food Institute (DTU, Denmark) facilities under conditions approved by the Danish Animal Experiments Inspectorate and the in-house Animal Welfare Committee.

Animal Sensitization Experiment. To study the sensitization capacity of intact Ara h 1, digested Ara h 1 and fractions of the digested Ara h 1, BN rats, 5−8 weeks of age, were allocated into five groups of 8−12 animals. Rats were immunized ip with PBS (control), 200 μg of intact Ara h 1, 200 μg of digested Ara h 1, or 200 μg of either the large or the small complexes, with the use of Alhydrogel 2% in PBS as adjuvant. Rats were immunized three times, at days 0, 14, and 28, and sacrificed at day 35 by exsanguination using carbon dioxide inhalation as anesthesia. For further details, see Bøgh et al.[10]

Enzyme-Linked Immunosorbent Assays (ELISAs) for the Detection of Specific IgG1, IgG2a, and IgE. ELISAs were performed as previously described.[10] Specific IgG1 and IgG2a were detected by direct binding of antibodies to plate-coated antigens, while IgE was detected in an antibody-capture ELISA, where Ara h 1 was coupled to digoxigenin.

Curve Calculations and Statistical Analysis. Curve calculations (XY analyses) and statistical calculations were made using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA). ELISA results expressed as antibody titers were examined for group differences, using the nonparametric one-way ANOVA, Kruskal−Wallis test, followed by Dunn's multiple comparison test for comparison of three or more groups. Differences between animal groups were regarded as significant when $P \leq 0.05$. Asterisks indicate a statistically significant difference between the given group and the control group. Asterisks over a horizontal line indicate a statistically significant difference between the two given groups: * = $P \leq 0.05$, ** = $P \leq 0.01$, and *** = $P \leq 0.001$

RESULTS

Characteristics of Purified Ara h 1. From N-terminal sequencing of the purified Ara h 1, it was evident that both known isoforms of Ara h 1 were present, in the ratio of approximately 1:1 (data not shown). The sequences identified were RHPPGER and RSPPGER, demonstrating that the purified isoforms of Ara h 1 start at amino acid residue 79 (RHPPGER, Ara h 1, clone P17, SwissProt no. P43237) or 85 (RSPPGER, Ara h 1, clone P41B, SwissProt P43238), a confirmation of a study by Wichers et al.,[33] showing that Ara h 1 is expressed as a truncated protein, in which the first 78 and 84 amino acids, respectively, are cleaved off. The endotoxin analysis of the purified intact Ara h 1 was <2 endotoxin units (EU)/mg of Ara h 1. From RP-HPLC analysis (Figure 2A), Ara h 1 was calculated to be >98% pure.

Characteristics of Digested Ara h 1 and Fractions Hereof. From RP-HPLC analyses, it was evident that no residual intact Ara h 1 was left in the three pools of Ara h 1 digests, since no detectable peak at the elution time for intact Ara h 1 was seen in the chromatography profiles (Figure 2A vs B−D). When comparing the RP-HPLC profiles for digested Ara h 1, large complexes and small complexes (Figure 2B−D), no significant differences are shown, indicating no apparent variation in peptide composition. However, when comparing

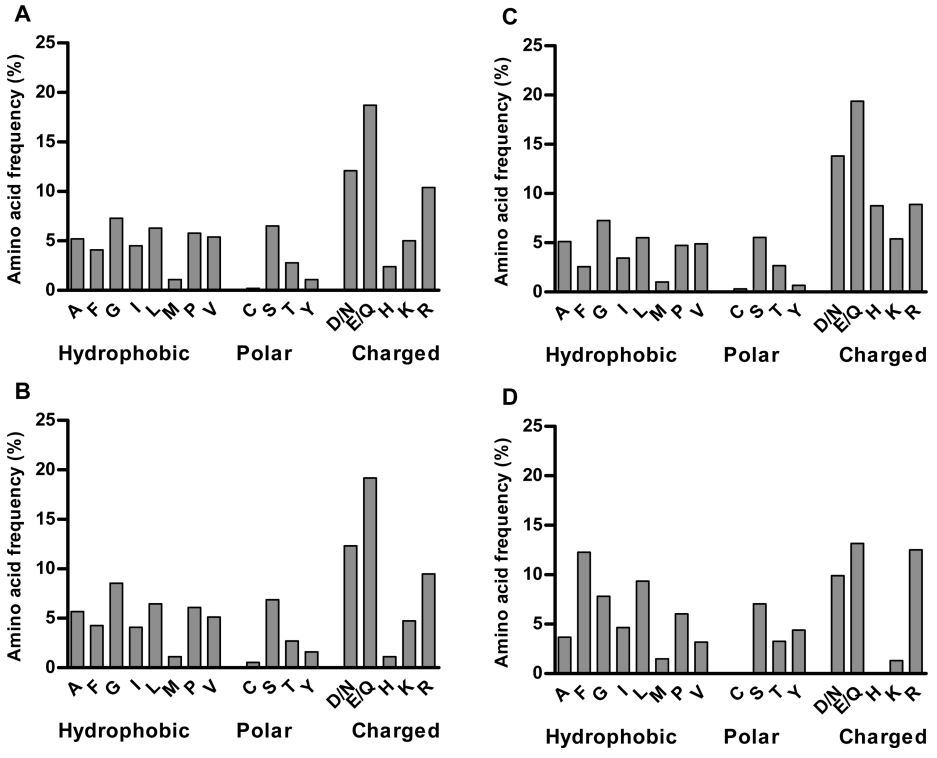

Figure 3. Amino acid frequency distribution. Comparison of the amino acid distribution for intact Ara h 1 (A), digested Ara h 1 (B), large complexes (C), and small complexes (D). The bars represent the frequency percentage of the indicated amino acid(s), represented by their one letter code, for either the intact Ara h 1 or all peptides represented in the different pools of Ara h 1 digests. Amino acids are grouped according to their physicochemical features.

the total amino acid distribution of the peptides present in the three different pools (Figure 3), it was revealed that differences did exist. While the amino acid distribution of digested Ara h 1 represented the amino acid distribution for intact Ara h 1, the large complexes and the small complexes were found to have an amino acid distribution different from that of the intact Ara h 1. That digested Ara h 1 had an amino acid distribution similar to the distribution of intact Ara h 1 confirms that this pool contains a peptide composition representative of the intact Ara h 1, where hydrophobic amino acids are responsible for approximately 40%, the polar for approximately 12%, and the charged for approximately 48% of total amino acids. In contrast, the large complexes contain approximately 35% hydrophobic amino acids, approximately 9% polar, and approximately 56% charged amino acids, while the small complexes contain approximately 48% hydrophobic amino acids, approximately 15% polar, and approximately 37% charged amino acids. This shows that peptides constituting the two fractions of digested Ara h 1 are different from each other and thereby different from the whole pool of digested Ara h 1 and, therefore, do not contain peptides representing the intact Ara h 1.

For examination of the peptide mass distribution profiles of the three different pools of Ara h 1 digests, MALDI-TOF MS was performed and demonstrated that Ara h 1 was digested to small peptide fragments of sizes $\leq M_r$ 4 000 (Figure 4), of which more than 75% had apparent M_r between 500 and 2 000. As in the whole pool of digested Ara h 1, the peptides in the large complexes had sizes up to M_r 4 000, while in the small complexes, the peptides were $\leq M_r$ 3,000. So while the digested Ara h 1 and the fraction of large complexes contained peptides that were up to 33 amino acids, the longest peptides in the fraction of small complexes were up to 25 amino acids. However, for all three pools of Ara h 1 digests, by far, most peptides were between 4 and 16 amino acids.

Realizing from the preparative GPC profile (Figure 1), based on which the fractions of Ara h 1 digests were made that the peptides were in some kind of aggregated state, we analyzed in more detail the aggregation profiles of the three different pools. From the analytical GPC profiles shown in Figure 5, it is evident that the peptides in all three pools were indeed aggregated into complexes of larger sizes. However, from the profiles, it is revealed that the state of aggregation is very different for the three different pools. From the area under the 220 nm GPC absorbance curves, it was indicated that 25% of the peptides in digested Ara h 1 were aggregated into complexes of up to M_r 104 000, that 53% of the peptides in the large complexes were aggregated into complexes of up to M_r 56 000, and that 7% of the peptides in the small complexes were aggregated into complexes of up to M_r 9 000. So, depending on the peptide composition profiles of the digested Ara h 1 and fractions hereof, the aggregation profiles differed significantly. See Table 1 for a summary of protein-chemical characteristics of the three different pools of Ara h 1 digests.

Sensitizing Capacity of Digested Ara h 1 and Fractions Hereof. Sera from individual BN rats dosed with either PBS (control), 200 μg of intact Ara h 1, 200 μg of digested Ara h 1, 200 μg of large complexes, or 200 μg of small complexes were evaluated for specific antibodies against both intact Ara h 1, digested Ara h 1, and fractions hereof. Looking at the antibody response, it was evident that while both intact Ara h 1 and whole pool of digested Ara h 1 could induce specific IgG response, neither the large complexes nor the small complexes could induce specific IgG antibodies. Analyses of the specific IgG1 (Figure 6) and IgG2a, which revealed similar

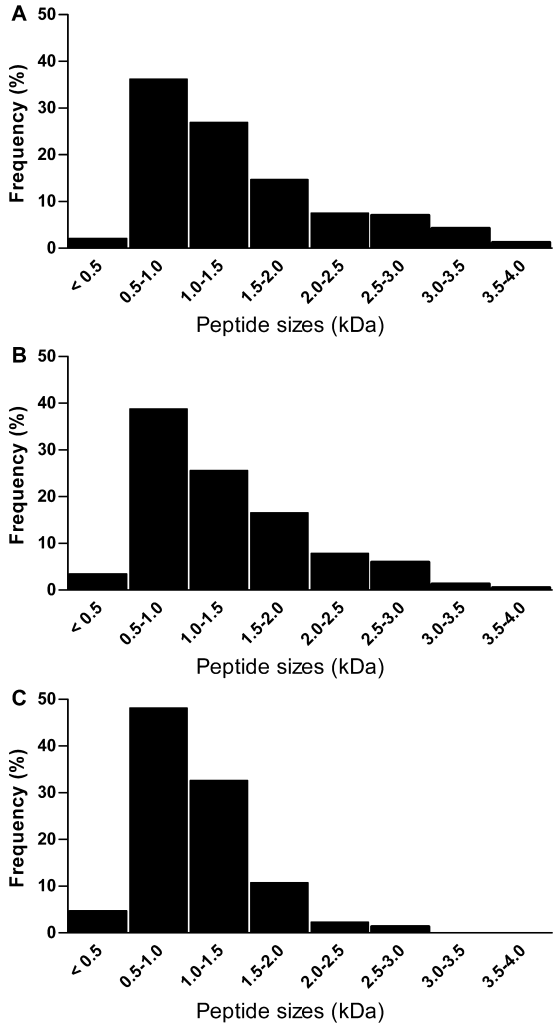

Figure 4. Peptide mass frequency distribution. Mass spectra of digested Ara h 1 (A), large complexes (B), and small complexes (C), shown in a histogram, where each bar corresponds to a peptide size interval of 0.5 kDa.

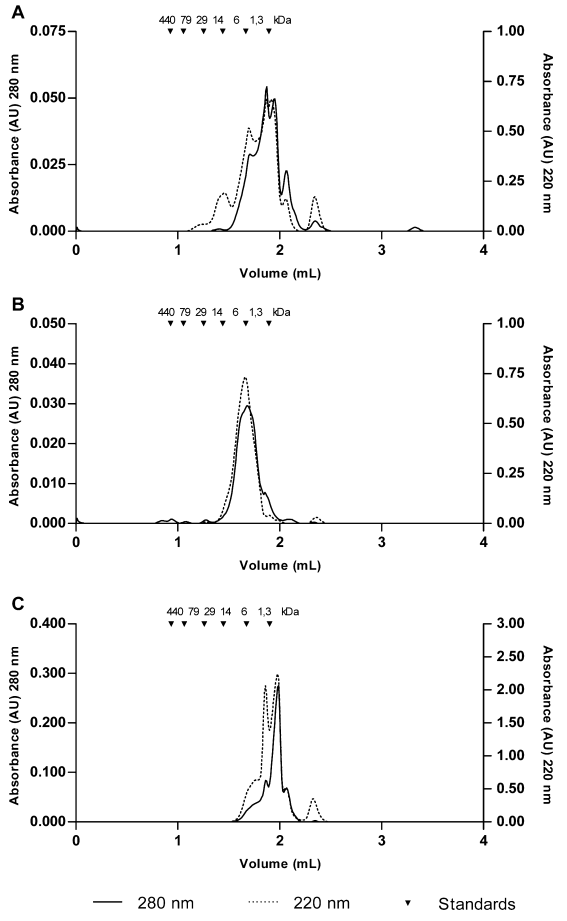

Figure 5. Analytical GPC. Comparison of chromatography profiles performed in 150 mM NH_4HCO_3 for digested Ara h 1 (A), fraction of large complexes (B), and fraction of small complexes (C), shown with absorbances at 280 and 220 nm. Standard MW markers for absorbances at 280 and 220 nm are shown across the top of the graph.

results (data not shown), showed that antibodies raised against intact Ara h 1 were able to recognize both intact Ara h 1, digested Ara h 1, and both fractions of the digested Ara h 1, all to a statistically significant level. Although it is seen from Figure 6 that all animals immunized with intact Ara h 1 could react with all four samples of allergens, it is seen that the binding capacity was different (although not statistically significantly). IgG1 antibodies from the rats immunized with intact Ara h 1 had the highest binding capacity toward the intact Ara h 1, followed by the whole pool of digested Ara h 1 and then the fraction of large complexes. The lowest binding capacity was toward the fraction of small complexes. Contrary to antibodies raised against intact Ara h 1, the antibodies raised against the whole pool of digested Ara h 1 could only react with intact Ara h 1 and the whole pool of digested Ara h 1, the latter being the only one that was statistically significant. The specific antibody responses showed no statistically significant differences between rats immunized with intact and digested Ara h 1.

From Figure 7, it is seen that both intact and digested Ara h 1 could induce specific IgE, although the intact Ara h 1-specific IgE response was only significant for antibodies raised against intact Ara h 1. However, no statistically significant difference was seen between rats immunized with intact and digested Ara h 1, respectively, by use of a multiple comparison test. Because it was not possible to couple the small peptide fragments in digested Ara h 1 to a coupling protein, which could be detected by commercially available secondary antibodies, we could not determine digested Ara h 1-specific IgE responses. We anticipate, however, that the specific IgE response of rats immunized with digested Ara h 1 would be higher for digested Ara h 1 than the one shown for intact Ara h 1. These speculations are based on our knowledge that specific IgE follows the specific IgG1 and IgG2a.[10,34]

DISCUSSION

The present study confirms that Ara h 1 retains both the sensitizing and the reacting potential, when digested to small peptide fragments. This signifies that digestion of Ara h 1 is not an effective approach for significant reduction of neither sensitizing nor IgE binding capacity and manifest that a correlation between resistance to digestion and allergenicity is not a general parameter. While Ara h 1 does not need to survive the digestion process as an intact protein or as large fragments to react with the immune system for induction of a specific immune response, this could still be the case for other food allergens. Previous studies examining the influence of digestion on the allergenic potential of other

Table 1. Overview of Protein-Chemical Characteristics of the Different Pools of Ara h 1 Digests

	amino acid distribution (%)				aggregation profile	
pool of digested Ara h 1	hydrophobic	polar	charged	peptide sizes (M_r)	amount (%)	sizes (M_r)
digested Ara h 1	40	12	48	≤4	25	104 000
fraction of large complexes	35	9	56	≤4	53	56 000
Fraction of small complexes	48	15	37	≤3	7	9 000

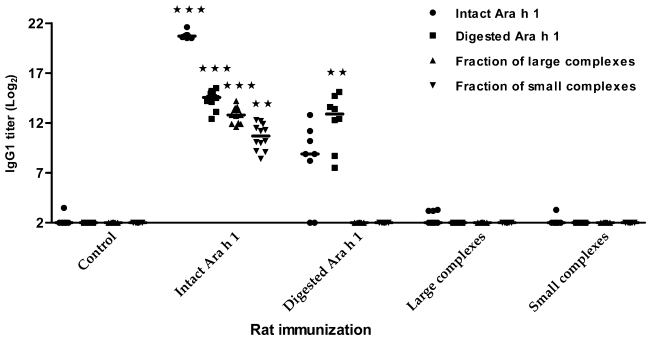

Figure 6. Specific IgG1 response. Comparison of specific IgG1 titer values, for groups of rats immunized with either PBS (control), intact Ara h 1, digested Ara h 1, large complexes, or small complexes. In each group of rats, IgG1 antibodies were examined for their binding to both intact Ara h 1, digested Ara h 1, the fraction of large complexes, and the fraction of small complexes. Each symbol represents an individual rat. Horizontal bars indicate the median value for each group of rats. The statistically significant difference between the groups was determined using Kruskal–Wallis test followed by Dunn's multiple comparison test. Asterisks indicate statistically significant difference of the given allergen-dosed group as compared with the control group for the given specificity (represented by identical symbols).

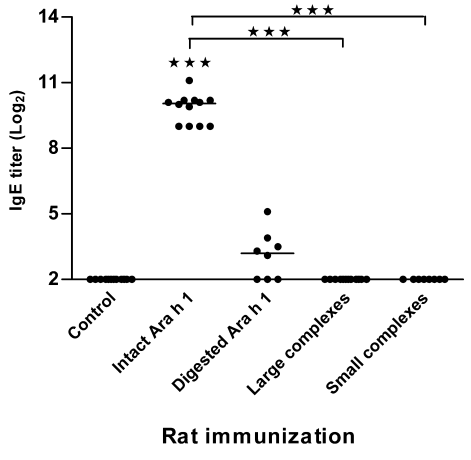

Figure 7. Specific IgE response against intact Ara h 1. Comparison of Ara h 1-specific IgE titer values, for groups of rats, immunized with either PBS (control), intact Ara h 1, digested Ara h 1, large complexes, or small complexes. Each symbol represents the specific IgE titer response toward intact Ara h 1 for an individual rat. Horizontal bars indicate median values. Statistically significant differences between groups of rats were determined using Kruskal–Wallis test followed by Dunn's multiple comparison test. Asterisks indicate statistically significant difference of the given allergen-dosed group as compared with the control group, and asterisks over a horizontal line indicate a statistically significant difference between the two given allergen-dosed groups.

food proteins have revealed digestion to be an effective approach for a significant impairment of sensitization[35,36] and IgE-binding capacity.[35,37,38]

We have previously shown that mixtures of peptides smaller than 2–5 kDa, which was generally thought to be the lower size limit for a peptide with inherent sensitizing capacity,[31] may still act as a "complete" allergen,[39] being able to sensitize, elicit allergic reaction, and react with IgE. The aim of this study was to further examine how small peptides retain their sensitizing capacity. This was done by studying the specific antibody responses in BN rats immunized with digested Ara h 1 and fractions hereof, separated on the basis of the aggregation profile of the peptides.

From the GPC analysis of the digested Ara h 1, it was evident that peptides did aggregate into complexes of larger sizes. This may be a result of noncovalent interactions, like hydrophobic interactions, since the single cysteine residue present in the Ara h 1 molecule cannot account for the amount and sizes of the complexes. That the sensitizing capacity of digested Ara h 1 is a result of the peptide fragments forming aggregates was hypothesized in earlier studies.[9,10] That aggregation may enhance the immune response toward antigen subunits was already recognized in 1978, where Morein et al.[40] showed that aggregation of subunits by hydrophobic interactions induced a significant higher immune response as compared to free subunits, suggesting an importance of multimeric structures. The same has been shown with the allergen melittin, a bee venom protein of 2.8 kDa (26 amino acids), with one B cell epitope[41] and one T cell epitope.[42] Melittin was able to induce specific IgG and IgE responses in humans and animals. The immunogenicity and allergenicity of the melittin were found to correlate with oligomerization of the molecules.[43−45] Also, the state of aggregation may influence the way in which proteins are presented to the immune system in the gastrointestinal tract. This was indicated in a study by Roth-Walter et al.,[46] which showed that aggregation of proteins from milk inhibited their uptake by intestinal epithelial cells and redirected uptake to Peyer's patches, promoting a significantly higher Th2-associated antibody response. This supports the theory that aggregation is a plausible explanation for the allergenic potential of digested Ara h 1.

It is well recognized that small peptides in general are poor immunogens and that peptides in general need to be of a certain size to behave as sensitizing allergens. Muller[31] stated that it is commonly assumed that peptides in the range of 2–5 kDa behave like haptens and are not immunogenic. It is known that for a protein to induce an allergic response, it requires the presence of both T and B cell epitopes. However, immunization with free peptides as small as 6–14 amino acid residues long has been reported to induce acceptable antibody responses.[31,47,48] This is in concordance with the earlier study of sensitizing capacity of digested Ara h 1, where peptides of less than 2 kDa were able to induce a statistically significant antibody response without the use of additional adjuvant.[10] This may indicate that the sensitizing capacity of digested Ara h 1 could also be an intrinsic feature of the free peptides themselves. It has been shown several times that induction of antibodies does not require covalent linkage between the

peptide behaving as T cell epitope and the peptide behaving as B cell epitope.[49−52] It has also been shown that amino acid sequences that did not function as an antibody epitope when part of a larger peptide were able to function as an antibody epitope when presented as free small peptides in mixtures with other peptides.[47,49] This is consistent with the present study showing that antibodies raised in rats immunized with the whole pool of digested Ara h 1 had higher binding capacity toward the digested Ara h 1 than toward the intact, indicating that new epitopes that were not accessible in the intact Ara h 1 became accessible when digested to small peptides. These results indicate that poor immunogenicity of peptides can be overcome by coimmunization with mixtures of the peptides.

To further elucidate why digested Ara h 1 retained the sensitizing potential, BN rats were immunized with fractions of the digest. Strikingly, this study showed that by separating the peptides of digested Ara h 1 into two fractions, the sensitizing capacity was lost. While only a small part of the peptides in the fraction of small complexes did aggregate, more than half of the peptides in the fraction of large complexes were in aggregates of sizes up to M_r 56 000. This is a larger part than in the whole pool of digested Ara h 1 where only 25% of the peptides aggregated. This reveals that allergenicity of the digested Ara h 1 is not simply a result of the peptides aggregating. The given fact that aggregated complexes in the digested Ara h 1 was up to M_r 104 000, nearly twice the size of the largest aggregates in the large complexes, indicates that the aggregation profile changed by means of fractionation in GPC. Instead of the sensitizing capacity being simply a result of aggregation of the peptides, the present study indicates that sensitization depends on the way the peptides do aggregate. A possible explanation may be that the aggregated peptides in the digested Ara h 1 are in an architecture representing the natural configuration of intact Ara h 1, while the peptides in the large complexes are in a state of de novo aggregation. Epitopes have been classified as either linear or conformational,[53,54] and it is believed that most B cell epitopes are conformational.[39,55,56] However, for food allergens, it has been suggested that linear epitopes could be of importance, since the protein is presented to the mucosal immune system of the gastrointestinal tract as denatured and digested protein fragments, favoring a B cell response toward linear sequences of amino acids.[57,58] Indeed, linear IgE-binding epitopes have been identified for various food allergens.[58] Nevertheless, linear IgE-binding epitopes have been found to contribute only a little to the total IgE binding,[53,54,56,59] and no biochemical characteristics were found to be shared between the linear IgE-binding epitopes.[58]

If sensitizing potential of digested Ara h 1 is an intrinsic feature of the free peptides themselves, however, coimmunization with B and T cell epitopes does not explain why the digested Ara h 1 retained sensitizing capacity, while this sensitizing capacity was abolished when the peptides were separated into fractions. An explanation could be that the stability of digested Ara h 1 by some means was changed when the peptides were separated. From amino acid analysis, it is evident that when the peptides of digested Ara h 1 were separated by GPC, the amino acid distribution was significantly changed, leaving most charged peptides in the fraction of large complexes and most hydrophobic peptides in the fraction of small complexes. Consequently, the same peptides may possibly not be present in the two different fractions and certainly not to the same extent. That the type and amount of peptides are of importance has been shown in several studies of peptide vaccine development. Mixtures of peptides have been shown to induce more B cell epitopes than did the very same peptides when fused or administered alone,[47,49,51,60] indicating that peptides may function as adjuvant or in a synergistic way. Accordingly, the present study suggests that most peptides in the digest need to be present to serve as adjuvant augmenting the immune response against other peptides and therefore need to be administered together. In addition, it has been demonstrated that peptides representing different T cell epitopes varied significantly in their ability to provide help to B cells. This suggests that the inherent property of the peptides constituting T cell epitope peptides differ in efficacy and that this feature may be independent of the protein from where they origin.[51,52]

The present study revealed that different requirements are needed for a protein to retain sensitizing and antibody binding capacity. While the fractions of digested Ara h 1 had no sensitizing potential, both fractions retained reactivity with antibodies raised in rats immunized with intact Ara h 1. This shows that there are larger requirements for peptides to sensitize than for the peptides to retain reacting activity. These results seem reasonable, since reacting activity only needs an amino acid sequence resembling an antibody epitope, while sensitizing capacity requires the ability to be recognized by the immune system de novo, priming of specific B cells as well as activation of T cells, providing the additional help needed for proper differentiation and proliferation of antibody secreting plasma cells. If most B cell epitopes of Ara h 1 are conformational, this leaves us to explain how antibodies directed against intact Ara h 1 are able to react with peptides from the small complexes. Peptides in the small complexes do not aggregate to an extent where they could represent conformational epitopes but must instead be epitopes derived from the linear sequence of the allergen. Aalberse[39] states that the main factor is the huge difference in binding affinity between antibodies interacting with intact protein versus interacting with peptides from the very same protein. This means that the peptides are much less efficient as compared to the intact protein for antibody binding. The peptide may for instance represent only a fraction of the epitope[39,54] or may only be a mimic of the epitope for which the antibody was originally directed against,[53] with only a certain degree of resemblance. The strength of interaction with the peptide could be decreased even more because of the higher flexibility of free peptides as compared to the complete protein.[39] This is in agreement with our own unpublished data, demonstrating a higher avidity between the binding of antibodies and intact protein as compared to the binding of the antibodies and digests. It is suggested that about 10% of antibodies directed toward conformational epitopes are able to react with linear peptide fragments of the protein,[53,54,56] which correlates well with the present study.

In summary, the current study showed that while digested Ara h 1 has sensitizing capacity, this capacity was lost after separation of the peptides in the digest into fractions. The sensitizing capacity of the digest was not dependent on single peptides but rather the sum of peptides. However, to unravel if the sensitizing capacity is a result of mixtures of free peptides or is a result of the peptides being in a defined aggregated state, further studies are needed. On the basis of the present study, we may conclude that the way in which the digests are presented to the immune systems is of significant importance

for the outcome and confirms the complexity of the mechanisms involved in sensitization.

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ABBREVIATIONS USED

ACN, acetonitrile; BN, Brown Norway; EU, endotoxin unit; GPC, gel permeation chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; MW, molecular weight; RP-HPLC, reverse phase high-performance liquid chromatography; SGF, simulated gastric fluid; TFA, trifluoroacetic acid

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PAPER 3

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IgE epitopes of intact and digested Ara h 1: a comparative study in humans and rats.

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Abstract

Background: Allergen epitope characterization provides valuable information useful for the understanding of proteins as food allergens. It is believed that IgE epitopes in general are conformational, nevertheless, for food allergens known to sensitize through the gastrointestinal tract linear epitopes have been suggested to be of great importance.
Objective: The aim of this study was to identify IgE specific epitopes of intact and digested Ara h 1, and to compare epitope patterns between humans and rats.
Methods: Sera from five peanut allergic patients and five Brown Norway rats were used to identify intact and digested Ara h 1-specific IgE epitopes by competitive immunoscreening of a phage-displayed random hepta-mer peptide library using polyclonal IgE from the individual sera. The resulting peptide sequences were mapped on the surface of a three-dimensional structure of the Ara h 1 molecule to mimic epitopes using a computer-based algorithm.
Results: Patients as well as rats were shown to have individual IgE epitope patterns. All epitope mimics were conformational and found to cluster into three different areas of the Ara h 1 molecule. Five epitope motifs were identified by patient IgE, which by far accounted for most of the eluted peptide sequences. Epitope patterns were rather similar for both intact and digested Ara h 1 as well as for humans and rats.
Conclusions: Individual patient specific epitope patterns have been identified for the major allergen Ara h 1. IgE binding epitopes have been suggested as biomarkers for persistency and severity of food allergy, wherefore recognition of particular epitope patterns or motifs could be a valuable tool for prevention, diagnosis, and treatment of food allergy.

1 Introduction

Peanut allergy is one of the most persistent and severe forms of food allergy (Emmett et al., 1999; Sicherer et al., 2001, 1999) and is mediated by allergen-specific IgE molecules, which bind to high affinity receptors on mast cells or basophils. Cross-linking of receptor-bound IgE by specific allergen, leads to degranulation and mediator release of the effector cells (Holowka and Baird, 1996; Lin and Sampson, 2009). For a food protein to be a 'complete' allergen (Aalberse, 2000, 1997) it must therefore be multivalent, i.e. expressing a minimum of two IgE-binding epitopes.
Since most IgE-binding epitopes are thought to be conformational (Aalberse, 2000; Barlow et al., 1986; Roggen, 2006; Van Regenmortel, 1996), the epitope profile of a globular protein is greatly influenced by the micro-environment. Thus, changes in e.g. pH, ionic strength, or binding to other molecules may affect the number of epitopes that are accessible for the antibodies by tightening or loosening the protein structure. In the latter case, new epitopes (neo-epitopes) may be observed. Such neo-epitopes are believed to be very prominent after proteolytic cleavage of the protein, as occur during digestion (Rich et al., 2001).
Different methods for identifying epitopes exist. Linear epitopes can be identified by analysis of IgE binding to peptides derived from the primary sequence (Burks et al., 1997; Lai et al. 2004; Roggen, 2006; Stanley et al., 1997). However, identification of conformational epitopes requires more elaborated methods, such as X-ray crystallography of antibody-antigen complexes (Niemi et al., 2007; Spangfort et al., 2003) or site-directed mutagenesis (Karisola et al., 2004; Lai et al., 2004; Spangfort et al., 2003). A promising alternative to these methods is competitive immunoscreening of a phage-displayed library of random oligopeptides to select epitope mimicking peptides followed by computer-based mapping of the identified peptide

sequences (Mittag et al., 2006; Roggen, 2006). Epitope mimics are small peptides, which mimics binding sites of the protein and are able to compete with the native protein for antibody binding (Meloen et al., 2000).

The peanut protein Ara h 1 is a major allergen, which is recognized by serum IgE from more than 80% of peanut allergic patients (Burks et al., 1997, 1991). In its native form Ara h 1 is a 63.5 kDa protein that forms stable homotrimers maintained by hydrophobic interactions between amino acids at the monomer-monomer contact points (Maleki et al., 2000; Shin et al., 1998). Both linear and conformational IgE binding epitopes of Ara h 1 have been mapped. Ditto et al. (2010) presented a case report supporting the presence of conformational Ara h 1-specific IgE epitopes. In addition, Ara h 1 are suggested to consist of 23 independent linear binding sites ranging from 6-10 amino acids in length (Burks et al., 1997) and a single linear binding site consisting of 25 amino acids (Shreffler et al., 2004), that have no obvious sequence motifs (a sequence pattern of amino acids) shared by them. Individual patients with IgE antibodies to Ara h 1 were shown to have IgE that recognized multiple epitopes on the Ara h 1 molecule, and of these epitopes four of them appeared to be immunodominant in that they were recognized by sera from more than 80% of the patients tested and bound more IgE than any other Ara h 1 epitopes. The 24 linear epitopes were evenly distributed along the entire primary sequence of the molecule (Burks et al., 1997; Shreffler et al., 2004). However, a molecular model of the tertiary structure of the Ara h 1 protein showed that the IgE epitopes were clustered into two main regions, the same regions where Ara h 1 monomer-monomer contact points are located (Maleki et al., 2000; Shin et al., 1998). Such reports, that the majority of the 24 linear IgE binding epitopes were suggested to be located in these hydrophobic contact sites led to the assertion that they may be protected from digestion, and that the quaternary structure of the Ara h 1 protein may play a significant role in its allergenicity (Maleki et al., 2000; Shin et al., 1998). Additionally, it is suggested that the structure may be so compact that potential cleavage sites are inaccessible until the protein is denatured and that the formation of a trimeric complex and further higher order aggregation may also afford the molecule some protection from protease digestion and denaturation (Shin et al., 1998).

Studies on the *in vitro* digestibility of Ara h 1 have produced rather divergent results. Becker (1997) reported that Ara h 1 was completely resistant to *in vitro* digestion. In contrast, Maleki et al. (2000) and Vieths et al. (1999) reported that it was digested but to relatively large MW peptide fragments only. Finally, the protein was reported to be easily digestible producing peptide fragments with maximum sizes as small as 2 kDa (Bøgh et al., 2009; Eiwegger et al., 2006; Kopper et al., 2004). The contradicting reports appearing in the literature is probably a result of different digestion conditions. However, although Ara h 1 was broken down to small peptide fragments, IgE binding epitopes survived the digestion process (Bøgh et al., 2009; Eiwegger et al., 2006). Digested Ara h 1 was shown to retain both sensitizing and eliciting capacity, which was suggested to be a result of the small peptide fragments aggregating to complexes of larger sizes (Bøgh et al., 2009). This notion is supported by a recent study, further indicating that the way in which the peptide fragments of digested Ara h 1 aggregate, is of major importance for the sensitizing capacity and suggesting that the peptide fragments is in an aggregated state resembling the intact Ara h 1 (Bøgh et al., 2012).

Susceptibility to digestion is an important issue since a characteristic feature of allergenic proteins in food is believed to be resistance to digestion (Astwood et al., 1996; FAO//WHO, 2001). Presently, it is recommended to include digestibility in risk assessment of novel proteins introduced into genetically modified food (FAO/WHO, 2001; EFSA, 2010).

This study was performed to investigate the impact of *in vitro* digestion on the IgE epitope profile of Ara h 1 as revealed by humans and rats. This was accomplished by competitive immunoscreening of a phage-displayed random peptide library using polyclonal IgE from individual patient and rat sera. The resulting

peptide mimics were mapped on the surface of a model of the 3-dimensional (3D) structure of the Ara h 1-monomer using a computer-based algorithm.

2 Material and Methods

2.1 Allergens

Ara h 1 was purified as described by Eiwegger et al. (2006), and digested as described by Bøgh et al. (2009). In short, purified Ara h 1 was digested in an *in vitro* gastro-duodenal model, designed to simulate the human digestion process, as described by Moreno et al. (2005a; 2005b). Digestion was performed with immobilized enzymes to make enzyme-free digests suitable for animal sensitization. The digestion process was divided into two phases, a gastric phase where intact Ara h 1 was digested with immobilized pepsin for 120 min at pH 2.5 and a duodenal phase where the gastric digests were further digested with immobilized trypsin and chymotrypsin for 15 min at pH 6.5.

2.2 Allergen characteristics

Purity of Ara h 1 was confirmed by reverse phase high-performance liquid chromatography to be > 99%.
Ara h 1 was digested to small peptide fragments, with sizes ≤ 2 kDa, of which more than 85% of the peptides had masses between 0.5 and 1.5 kDa, demonstrated by mass spectrometry. From gel permeation chromatography it was indicated that more than 50% of the peptide fragments occurred in aggregated complexes of up to M_r 20.000, as described in Bøgh et al. (2009).

2.3 Peanut allergic patient sera

Sera from five individual patients (patient no.: 2205, 2208, 2209, 2304, and 2305) with clear-cut peanut allergy diagnosed by an experienced allergologist were used. These patients had convincing history of peanut anaphylaxis (severe, potentially fatal, systemic allergic reaction that occurred suddenly after contact with peanut) or clear peanut exposure-related symptoms (urticaria, itching of the skin, gastrointestinal symptoms) within the last 12 months (Table 1). Due to severity and clear-cut exposure-related recent reactions a DBPCFC was not justified from an ethical point of view.
All sera were tested positive for peanut extract-specific IgE by ImmunoCAP and Ara h 1-specific IgE by ELISA (Eiwegger et al., 2006). The protocol was approved by the local ethical committee of the Medical University of Vienna and written informed consent was obtained (protocol no° EK 428/2008).

2.4 Brown Norway rat sera

Sera from five individual Brown Norway (BN) rats immunized three times i.p. with either 200 µg purified intact Ara h 1 (rat no.: 49, 52, 53, and 54) or 200 µg gastro-duodenal digestion products hereof (rat no.: 19) without the use of adjuvant were used. The BN rats were kept on a diet free from *leguminosa* for at least three generations.
All sera were tested positive for intact and digested Ara h 1-specific IgE by ELISA and Rat Basophilic Leukaemia (RBL)-assay. From the RBL-assay it was also evident that IgE was biologically functional being able to induce degranulation of RBL cells with both intact and digested Ara h 1 (Bøgh et al., 2009). Animal experiments were carried out at the National Food Institute (DTU, Denmark) facilities and performed under conditions approved by the Danish Animal Experiments Inspectorate and the in-house Animal Welfare Committee.

2.5 Selection of phage-displayed IgE epitope mimicking peptides

IgE epitope mimicking peptides were identified by competitive immunoscreening of phage-displayed libraries of random oligopeptides essentially described by Mittag et al. (2006), which allows for detection of both linear and conformational epitopes. In short, four x 500 µL aliquots of tosyl-activated M280 Dynabeads suspension (Dynal Biotech, Olso, Norway) were each coated with 25 µg rabbit-α-human IgE (DAKO, Glostrup, Denmark) or mouse-α-rat IgE (Oxford Biotechnology, Kidlington, UK) for 48 h at 37 °C, and blocked with 0.5 % (w/v) skimmed milk powder (SMP) in Phosphate Buffer Saline (PBS). Patient serum (1000 µL) or rat serum (500 µL) was diluted in PBS with 0.5 % SMP (w/v) and 0.05 % (w/v) Tween 20 (dilution buffer), and incubated overnight at 4 °C with 400 µL or 200 µL coated bead suspension, respectively. After extensive washing, 2 x 50 µL (for human) or 2 x 25 µL (for rat) of the coated beads were used immediately for the first round of selection, while the remaining 300 µL or 150 µL, respectively, were restored for later selection rounds.

2.5.1 ***First selection round***

Fifty µL or 25 µL beads with immobilized IgE were blocked in 1 mL of 2% (w/v) SMP in PBS for 1 h at room temperature (RT) and washed extensively. The beads were then incubated overnight with 10 µL (~2 x 10^{11}) phages from a library of phages displaying linear random hepta-mer peptides (Ph.D.-7 Phage Display Peptide Library Kit, New England BioLabs, Beverly, MA, USA), followed by extensive washing and negative selection to remove unbound phages. For positive selection, phages were eluted by competitive immunoscreening, by adding 25 µg allergen (intact or digested Ara h 1). Eluted phages were amplified by direct infection of ER2738 *E.coli* cells (provided with the phage library). Phage amplification was allowed overnight at 37 °C. Amplified phages were purified by PEG/NaCl-precipitation and tittered.

2.5.2 ***Second selection round***

Fifty µL or 25 µL beads with immobilized IgE were incubated with ~10^{9}-10^{10} amplified phages selected in the first round. All further steps were performed as described for the first selection round.

2.5.3 ***Third selection round***

Third selection round took place as described for the second, besides that eluted phages were not amplified right away. Instead phages were tittered and single colonies were picked, isolated and amplified as described by the library manufacturer.

2.6 Phage-capture ELISA for determination of IgE-reactivity to phage-displayed peptides

For selection of positive phage clones microtitre plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4˚C with 100 µL/well of 8.3 µg/mL rabbit-α-human IgE (DAKO, Glostrup, Denmark) or 100 µL/well of 1.0 µg/mL rabbit-α-rat IgE (Zymed, Berlin, Germany) in PBS. All following steps were performed at RT for 1 h. Plates were blocked with 200 µL/well of 2% (w/v) SMP in PBS. Between each step plates were washed 3 times in PBS with 0.05% (w/v) Tween 20 (PBS-T). One half of the plate was incubated with 100 µL/well of serum diluted 1:60 (human) or 1:20 (rat) in dilution buffer while the other halve of the plate was incubated with 100 µL/well of dilution buffer (background). For plates with rat serum, plates were blocked once more, this time with 200 µL/well of naïve rat serum diluted 1:20 in dilution buffer. Sequently, all plates were incubated with 100 µL/well supernatants from single colonies of amplified phages or wild type phage without an insert coding for a peptide (negative control), followed by incubation with 100 µL/well of mouse-α-M13 phage (Amersham Biosciences UK Limited, Buckinghamshire, UK) diluted 1:1000 in dilution

buffer. For detection, the plates were incubated with 100 µL/well of alkaline phosphate (AP)-conjugated goat-α-mouse Ig (DAKO, Glostrup, Denmark) diluted 1:1000 in dilution buffer. Phage-IgE interactions were visualized with 100 µL/well of 1 mg/mL *p*-NitroPhenyl Phosphate (Sigma, Berlin, Germany) in diethanolamine buffer as AP-substrate. Incubation was conducted at 37 ˚C for 30 min. Reaction was stopped by adding 100 µL/well 1.0 M NaOH. Absorption was measured at 405 nm with a microtitre reader (Molecular Devices, Sunnyvale, CA, USA).
The results were corrected for background, where OD values of greater than OD + 3 x S.D. of the negative control were regarded as a positive result.

2.7 Phage DNA precipitation and sequencing

Precipitation of DNA from the selected phages was carried out according to manufacturer instruction. DNA sequencing of the displayed hepta-mer peptides was performed using M13 phage specific primers.

2.8 Mapping of peptides on the Ara h 1 monomer surface using 3D-structure

Amino acids corresponding to the selected peptides were localized on the 3D structure of the Ara h 1 monomer (SwissProt no. P43237) amino acid 164-583. (The homology based model of Ara h 1 amino acid 164-583, was constructed from the X-ray crystal structure of canavalin from jack bean (Ko et al., 2000), which have a sequence identity with Ara h 1 of 48%, and optimized to give lowest Gibbs free energy (G). The peptides obtained by immunoscreening of the phage-displayed peptide library were localized on the Ara h 1 surface using a computer-based algorithm (Epitope mapping tool (EMT), Novozymes A/S, Bagsvaerd, Denmark) as described by Batori et al. (2006). The potential epitope mimics on the Ara h 1 molecule were subjected to structural analyses using Swiss-PdbViewer (http://www.expasy.org/) to remove those that were unlikely to occur due to severe structural constraints. By mapping the selected peptides on the 3D structure of industrial enzymes no localization of epitope mimics were possible.

3 Results

The IgE binding regions of intact and digested Ara h 1 were assessed by competitive immunoscreening of a phage-displayed random hepta-mer peptide library using polyclonal IgE from five individual peanut allergic patients and five individual BN rats immunized with either intact or digested Ara h 1. The resulting peptide mimics were mapped on the surface of a model of the 3D structure of the Ara h 1-monomer using a computer-based EMT. The obtained data were analyzed in order to identify quantitative and qualitative differences between the epitope profiles of intact and digested Ara h 1, as well as between the profiles for humans and rats.

3.1 Identification of IgE binding epitope mimics

3.1.1 *Peanut allergic patients*

A total of 123 IgE binding peptide mimics were selected for the five individual peanut allergic patients. The number of mimics was rather similar (N = 20-32) for all patients, and (N = 8-17) for both intact and digested Ara h 1. Some of the peptide sequences were eluted multiple times for an individual patient, some eluted with both intact and digested Ara h 1, and some shared by two or more peanut allergic patients (Table A.1). Each patient was found to have an individual pattern of eluted sequences, which was independent of the elution condition (i.e. if eluted using either intact or digested Ara h 1). However, five motifs (a sequence

pattern of amino acids) were found to account for more than 65% of all eluted sequences; K*PAF*L, PRG[I/L/V]F, SPI*LY, DRGLF and PYTL[D/S]K (Table 2). As can be seen from the Table 2, the recognition of these five motifs was not equally distributed between the five peanut allergic patients. While the motif K*PAF*L and motif PYTL[D/S]K were only eluted for a single patients the motif DRGLF were eluted for four patients. While the patients 2208, 2209 and 2305 recognized three of the five motifs, patient 2205 only recognized one motif. Also the proportion of eluted sequences corresponding to a motif varied between the five patient, where all eluted sequences for patient 2205 corresponded to a single motif, less than half of all eluted sequences corresponded to a motif for patient 2305. This demonstrates heterogeneity of the Ara h 1-specific IgE antibody response between patients, which yet seems to overlap to a significant degree for some of the patients. However, the absence of a motif recognition for a patient does not exclude that such motif could not be recognized by the patient but only implies that the IgE antibody-affinity for this motif was not high enough to be selected for sequencing.

Importantly form the Table 2 it is shown that all five motifs were eluted with both the intact and the digested Ara h 1, revealing the survival of these epitope motifs after simulated gastro-duodenal digestion.

3.1.2 BN rats

A total of 138 IgE binding peptide mimics were selected for the five individual rats. The number of mimics was rather similar (*N* = 23-38) for all rats, as well as was the number (*N* = 11-22) for intact and digested Ara h 1, respectively. (Rat no 52 only had sequences eluted with intact Ara h 1 because of serum shortness) (Table. A.2). Some of the peptide sequences were eluted multiple times for an individual rat, some eluted with both intact and digested Ara h 1, and some shared by two or more rats. However, each rat was found to have an individual pattern of epitope mimics, which was irrespective of the immunization and eluting condition (intact vs. digested Ara h 1).

3.2 Amino acid distribution of the epitope mimics

To identify if any qualitative differences in amino acid composition of the found epitope mimics existed between intact and digested Ara h 1 and between humans and rats, an analysis of the amino acid distribution was performed. No apparent difference in overall physico-chemical characteristics of the amino acids involved in the epitope mimics between intact and digested Ara h 1 and between humans and rats was observed (data not shown). Hydrophobic amino acid residues occurred most frequently, responsible for over 50% of all amino acids in the identified epitope mimics, with polar residues being the next most frequent type of amino acids, responsible for around 30%, and charged residues representing the least frequent amino acid group containing around 16%. This was applicable for both eluting conditions as well as both immunization conditions (intact and digested Ara h 1), and for both human and rat specific epitope mimics. This amino acid distribution according to physico-chemical characteristics, is not a picture of the general amino acid distribution of the Ara h 1 molecule (SwissProt no. P43237; amino acid 79-614, which is the full length of the purified Ara h 1 molecule after cleavage of the N-terminal (Bøgh et al., 2012)), where the hydrophobic amino acids account for 40.6%, the polar for 24.6%, and the charged amino acids for 34.6% of all amino acids. This indicates that the epitope mimics are clustered into the more hydrophobic and less charged areas of the Ara h 1 molecule.

Differences in amino acid distribution, according to single amino acids, between sequences eluted with intact and digested Ara h 1 and between humans and rats (Fig. 1A-F), are small, especially when compared to the general amino acid distribution for the Ara h 1 molecule (Fig 1G). The most common amino acids in the eluted sequences were Leucine (L), Proline (P), and Serine (S) for both humans and rats. In contrast the most common amino acids in the entire Ara h 1 molecule are Glutamic acid (E) and Arginine (R). In fact Glutamic acid, which are the most common amino acid in the Ara h 1 molecule, occurred only seldom in the

eluted sequences. Comparing amino acid distribution according to immunization condition in the rats (intact vs. digested Ara h 1) (Fig. 1E-F) small differences are evident. These are most pronounced for the amino acid Alanine (A) and Serine (S).

3.3 Ara h 1 IgE binding fingerprinting profile

The Ara h 1 epitope fingerprinting profile, defined as the frequency distribution of amino acid residues identified as being involved in IgE binding along the primary protein sequence is shown in Fig. 2. From the IgE binding epitope fingerprinting profiles it is evident that some amino acid residues along the primary sequence of the Ara h 1 molecule appear more often than others in the suggested mapped epitope mimics. This indicates that some regions of the primary structure is more likely than others to be involved in IgE binding and that amino acid residues being part of the IgE epitopes cluster into specific areas. Some amino acid residues account for nearly 5% of all specific amino acids found in IgE binding sequences and other amino acid residues did not occur in any mapped epitope mimics at all.

When comparing the IgE binding profile for Ara h 1 according to eluting condition (intact vs. digested Ara h 1), it is seen that the profiles are very similar, for both patients and rats, respectively (Fig. 2A-D). A similar pattern is seen for both peaks and gaps, which are lying in the same areas of the Ara h 1 primary structure. It was also seen that most amino acids occurring in the epitope mimics are clustered, according to primary structure, between amino acid residue 220 and 270.

When comparing the Ara h 1 IgE binding fingerprinting profile according to immunization condition (intact vs. digested Ara h 1) for rats (Fig. 2E-F), it is seen that the profiles are different from each other. This is primarily shown by the larger gaps in the fingerprinting profile obtained for the rat immunized with digested Ara h 1. Any conclusions should however be taken with caution, since the results are based on a single rat immunized with digested Ara h 1.

Comparing the Ara h 1 IgE binding fingerprinting profiles for humans and rats (Fig. 2A-D) it is seen that the profiles are very similar, with peaks in the same areas of the primary structure of Ara h 1, though differences in frequency for single amino acid residues occur.

3.4 Mapping of epitope mimics on the surface of Ara h 1

3.4.1 *Peanut allergic patients*

Computer-based mapping of the eluted sequences on the 3D structure of the monomer Ara h 1 (SwissProt no.P43237, amino acid 164-583) revealed that all sequences corresponded to conformational epitopes, although short stretches of juxtaposed amino acids were found in some mimics.

Irrespective of whether the sequences were eluted with intact or digested Ara h 1, the mapped epitope mimics clustered into three areas when based upon those amino acid residues which occurred in at least 3% of all mapped IgE epitope mimics. This cut-off corresponds to specific amino acids occurring four or more times in all epitope mimics. Most epitope mimics were thus clustered in the yellow area (Fig. 3.). So in consistency to identification of the localization along the primary sequence of Ara h 1 (Fig. 2.), it is also here identified that the epitope mimics are not distributed equally throughout the entire molecule but seems to cluster into some areas.

Mapping of the eluted heptameric sequences on the surface of Ara h 1 showed that the epitope mimics were well exposed on the molecular surface of the 3D model of an Ara h 1 molecule and that no significant differences was evident between the elution with intact or digested Ara h 1 (data not shown).

3.4.2 *BN rats*

As was the case for the peanut allergic patients, only peptide sequences corresponding to conformational epitope mimics emerged from the selection. For a few of these sequences more than one good match could be found on the surface of the Ara h 1 model.

Even though, each rat was found to have an individual pattern of epitope mimics, most IgE selected peptides were shown by the EMT to be localized into the same three surface areas of the Ara h 1 molecule, as were the peptides selected with the patient sera. See Figure 3. However it was observed that no motifs was shared by the eluted peptide sequences, which was in contrast to the results found for the peanut allergic patients.

With regard to the accessibility of the epitope mimics on the surface of an Ara h 1 molecule, also for the rats no significant differences were seen between the elution with intact and digested Ara h 1, respectively (data not shown).

4 Discussion

This study supports the notion that most IgE binding epitopes are conformational and not linear in nature (Aalberse, 2000; Barlow et al., 1986; Roggen, 2006; Van Regenmortel, 1996). Although we used a method able to detect both linear and conformational epitopes, we only identified sequences (regardless of human or rat) mimicking epitopes on Ara h 1 which were composed of amino acid residues put together by the tertiary structure of the molecule or by two or more short stretches of juxtaposed amino acid residues. This is in contrast to most of the previous studies of Ara h 1-specific IgE binding epitopes showing that these are linear in origin. Burks et al. (1997) identified a total of 23 IgE binding epitopes consisting of 6-10 amino acids and Shreffler et al. (2004) identified yet another consisting of 25 amino acid, using the methods of overlapping peptides, which allows for detection of only linear epitopes, to identify the IgE binding sites. Additional studies confirmed the importance of such linear Ara h 1-specific IgE binding epitopes (Beyer et al., 2003; Flinterman et al., 2008; Shin et al., 1998). This is in agreement with the suggestion that linear IgE epitopes could be of special importance for food allergens known to sensitize through the gastrointestinal tract (Bannon and Ogawa, 2006; Lin and Sampson, 2009; Pomes, 2010).

The present study indicates that there could be five important conformational IgE binding epitopes on Ara h 1, since more that 65% of all eluted sequences, based on patient sera, could be categorized as belonging to one of only five motifs. The five motifs showed patterns of a minimum of five amino acids, which is also the number of amino acids suggested to be involved in binding between antibody and antigen. Even though epitopes are suggested to consist of at least 8 amino acids, energy calculations suggest that a smaller subset of 5-6 amino acids are the key contributors to the binding between antibody and epitope (Bannon and Ogawa, 2006; Laver et al., 1990; Van Regenmortel, 1996). When choosing the use of short heptameric peptides for competitive immunoscreening and selecting only the eluted sequences showing the highest affinity in ELISA, we are in favor of selecting only those peptides containing the essential amino acids contributing to the binding between IgE molecules and epitopes, and deselect those which could simply be a result of non-specific binding. Also we are in favor of selecting only those peptides representing epitopes with the highest affinity to the specific IgE antibodies, while deselecting those with lower affinity. Therefore, the absence of an epitope mimic for one of the patients does not necessarily imply that this epitope could not be recognized by the patient. However, that 65% of all eluted sequences could be adjusted to belong to only five motifs and that these five motifs were identified not only with one patient

and with one eluting condition, indicate that these motifs could be very important for the allergenicity of Ara h 1. Indeed all five motifs were identified by both intact and digested Ara h 1, and three of them were identified by three or four patients.

Motifs were only identified by humans and not rats. Rats showed a much more heterogeneous pattern and only few eluted sequences were shared by two or more rats, and a greater proportion of the complete allergen surface seemed to be involved in mimicking IgE binding sites. This indicated that in contrast to humans no or only little affinity-maturation had occurred in rats. This, however, seems reasonable since the allergic history of rats consisted of only 5 weeks in contrast to patients which had a long and consistent history of peanut allergy. Arnon and Van Regenmortel (1992) and Van Regenmortel (2009) states that epitopes are not an intrinsic characteristic of an allergen that exists independently of its antibody partner. It is estimated that by immunization of an individual with a protein-antigen, more than 100 different antibodies will be formed, which differ in their epitope-specificity and may be directed against partly overlapping epitopes, so that the entire accessible surface of an allergen harbors many overlapping epitopes (Kawamura et al., 1984; Schroer et al., 1983). Upon reexposure to the same allergen affinity-maturation occurs, where progressively higher affinity B cell clones are generated, as a result of somatic hypermutation and antigen-specific selection of high affinity B cells (Griffiths et al., 1984; Kocks and Rajewsky, 1988; Neuberger et al., 2000; Siskind and Benacerraf, 1969). Another explanation for the many eluted sequences seen for the rats, could be a result of 'true' cross-reaction. As the relationship between an antibody and an epitope is not of an exclusive nature, 'true' cross-reactivity may occurs, where an antibody reacts with other epitopes (here, eluted sequences) that is different in structure, but related to the epitope, which the particular antibody was originally raised against (Arnon and Van Regenmortel, 1992). This could in particular be relevant in situations where affinity maturation have not been fully established. Only a limited degree of resemblance is suggested to be sufficient to allow cross-reactivity (Arnon and Van Regenmortel, 1992; Van Regenmortel, 2009).

Besides the occurrence of motifs the epitope mapping profile of humans and rats were very similar. Both the IgE binding fingerprinting profile, amino acid distribution and the localization and clustering of epitopes on the Ara h 1 molecule were similar, indicating that humans and BN rats direct their antibody-response against the same areas of the allergen. This is one important issue in identifying a suitable animal model (Knippels and Penninks, 2005), and has also been shown in an earlier study comparing human and BN rat allergic responses (Miller et al., 1999).

The definition of epitope seems to be a little unclear, so instead of talking about specific epitopes, it would make more sense to talk about epitope areas. The same amino acid residues in a protein can be part of different overlapping epitopes recognized by different antibodies, which is also evident from this study. Therefore, as Van Regenmortel (2009) states, it is not possible to draw clear boundaries between individual epitopes and there is no clear definition of an epitope on e.g. the atomic composition of binding interface to the antibody or the binding affinity that can be used as an benchmark to decide what is an epitope or if two epitope are the same or not. In light of this it seems even more important for the validation of the BN rat model that epitopes were clustered into the same areas of Ara h 1. Earlier studies have also indicated that Ara h 1-specific IgE epitopes could be clustered into areas of the monomeric Ara h 1 molecule (Maleki et al., 2000; Shin et al., 1998). Clustering of IgE epitopes have also been shown for Bos d 4 (Hochwallner et al., 2010), Phl p 1 (Flicker et al., 2006), Bet v 1 (Fedorov et al., 1997), and Phl p 5 (Flicker et al., 2000), and have been suggested to be a general phenomenon for allergens determining the allergenic activity (Hochwallner et al., 2010; Flicker et al., 2006).

In the present study we found that the IgE epitope profiles of intact and digested Ara h 1 were very alike, as the fingerprinting profile, amino acid distribution, localization and clustering of epitopes on the monomeric Ara h 1 molecule came out similar when elution was made with intact or digested Ara h 1, respectively. It seems especially important that all five identified motifs were identified when elution was made with both intact and digested Ara h 1. This indicates that even though Ara h 1 is digested to very small peptide fragments IgE binding epitopes survived the digestion process. This is in agreement with our earlier studies, showing that both intact and digested Ara h 1 were able to sensitize BN rats and elicit a biological relevant response (Bøgh et al., 2009, Bøgh et al., 2012). Ara h 1 is a labile protein digested to peptides of ≤ 2 kDa, which correspond to around 17 amino acids. However, more than 50% of these peptides were aggregated to complexes of sizes up to 20 kDa. Aggregation of Ara h 1-digests is suggested to be the reason for the retained allergenicity of the digested Ara h 1 (Bøgh et al., 2009). That the peptides are in aggregated form under physiological conditions make the survival of conformational epitopes possible. Further, it is suggested that the digested Ara h 1 are kept in a conformation resembling the native structure of Ara h 1 (Bøgh et al., 2012), probably by non-covalently interactions, such as hydrophobic interactions. It has been shown that the hydrophobic amino acids of the Ara h 1 molecule are important for the IgE binding (Maleki et al., 2000; Shin et al., 1998). This is consistent with our findings showing that the amino acid residues composing the epitopes are relatively hydrophobic compared to the general Ara h 1 monomeric molecule. That conformational epitopes of a labile food allergen are able to survive digestion has not been shown before. Since most B cell epitopes are thought to be conformational and the fact that we find a significant cross-reactivity between intact and digested Ara h 1, supports the hypothesis that the peptides in digested Ara h 1 are in an aggregated form which represents the original folding of the intact Ara h 1.

The eluted epitope mimics are localized on a 3D model of Ara h 1 (amino acid 164-583), which is made by homology modeling of canavalin (Ko et al., 2000), and is therefore shortened in both N- and C-terminal ends. This makes it possible that some epitopes are missed. Also sequences obtained with peptide libraries are not necessarily real epitopes but only a mimic of epitopes. However, the level of mimicry is significant since an assessment of binding activity is the foundation for the selection of epitope mimics in the study. Since the epitope-specificity of an antibody is never of completely unique nature, considerable cross-reactivity may occur and inherent multispecificity of antibodies may exist (Arnon and Van Regenmortel, 1992; Van Regenmortel, 2009). The identified epitope matches on the Ara h 1 surface is not a precise identification but more an attempt to indicate the most likely location of the epitopes. Identification of IgE-binding epitopes is an important issue in allergic diseases, where it can be used for a better understanding of the immune response. Epitope profiles can be useful for studying potential cross-reactivity between homologous proteins (Pomes, 2010), for design of recombinants for immunotherapy (Karisola et al., 2004), for design of peptide vaccines (Hochwallner et al., 2010), in a predictive manner when assessing allergenic potential of novel foods and genetically modified foods, and as a predictive biomarker for persistency, severity or diagnostic treatment of an allergic disease (Lin and Sampson, 2009; Huang and Honda, 2006). Indeed, different IgE epitope patterns have been found to be associated with the allergic phenotype, where e.g. epitope specificity correlated with persistency and diversity correlated with severity (Lin and Sampson, 2009). The present described epitope mapping technique could be a useful tool in allergic diseases for studying motif identification and pattern recognition by IgE.

Acknowledgements

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

3D, 3-dimensional; A, anaphylaxis; AP, alkaline phosphate; BN, Brown Norway; BO, bronchial obstruction; D, digested; EMT, epitope mapping tool; F, female; GI, gastro-intestinal symptoms; I, intact; M, male; OE, oral oedema; PBS-T, PBS Tween 20; RBL, Rat Basophilic Leukaemia; RPB, reduced blood pressure; RT, room temperature; SMP, skimmed milk powder; U, urticarial

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Figure legends

Fig. 1. Amino acid distribution in IgE binding sequences. The bars represent the total number of a particular type of amino acid residue found in all of the eluted IgE binding peptides. Amino acid distribution is represented as overall frequency of a single amino acid residue for a given condition: Amino acid distribution based on patient sera for peptide sequences eluted with intact (A) or digested (B) Ara h 1, or amino acid distribution based on sera from BN rats for sequences eluted with intact (C) or digested (D) Ara h 1, or amino acid distribution based on BN rats immunized with either intact (E) or digested (F) Ara h 1, and the general amino acid distribution for the expressed Ara h 1 molecule (G). The amino acids are grouped according to their physico-chemical feature.

Fig. 2. Ara h 1 IgE binding epitope fingerprinting profiles. Epitope frequency is represented as sums of each amino acid residue along the primary protein sequence (amino acid 164-583) found in all suggested mapped epitope mimics in a given condition: For peanut allergic patients, where sequences were eluted with intact (A) or digested (B) Ara h 1, for rats where sequences were eluted with intact (C) or digested (D) Ara h 1, and for rats immunized with intact (E) or digested (F) Ara h 1.

Fig. 3. Ribbon diagram of the 3D structure of the Ara h 1 monomer. Localization of the IgE binding areas characterized by the polyclonal IgE response of the five peanut allergic patients. When cut off level for frequency of single amino acid residues in the suggested epitope mimics was set to four, it was seen that the epitope mimics were clustered into three epitope areas on the surface of the Ara h 1 molecule, indicated by the yellow, red, and blue regions. By far the majority of the epitope mimics were clustered into the area indicated by yellow colour. The figures are views of the Ara h 1 molecule by means of 180° rotation about a vertical axis.

Tables

Table 1.

Patients' characteristics.

ID	Anti-whole peanut IgE (IUA/L)	Anti-Ara h1 IgE (IAU/L)	Total IgE (kU/L)	Clinical symptoms after peanut consumption	Age	Gender
2205	45	29	535	A, OE, BO, GI	25	M
2208	18	10	26.9	A, OE, GI	23	M
2209	250	91	>2000	A, RBP, BO	27	F
2304	327	262	1013	A, BO, GI	25	M
2305	155	129	>2000	A, RBP, OE, U, GI	18	F

A: anaphylaxis, BO: bronchial obstruction, GI: gastro-intestinal symptoms, OE: oral oedema, RBP: reduced blood pressure, U: urticaria, M: male, F: female

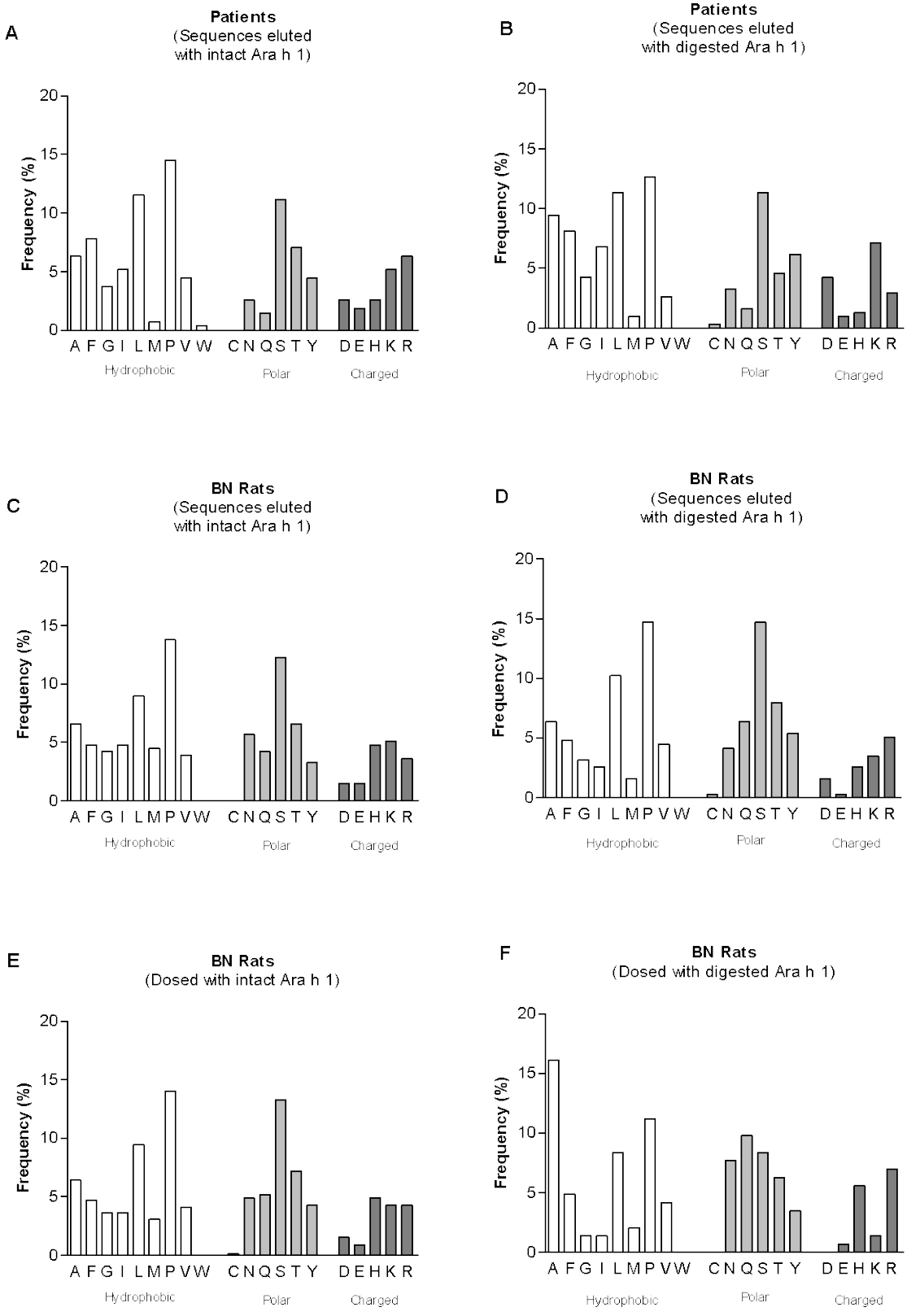
Table 2.

The five motifs found in the epitope mimics for the five peanut allergic patients.

Motifs	Localization of the Ara h 1 monomer molecule	Proportion of sequences eluted with either intact (I) or digested (D) Ara h 1, belonging to the given motif for each individual patients				
		2205	2208	2209	2304	2305
K*PAF*L[(a)]	$K^{431}XP^{512}A^{514}F^{577}XL^{399}$ $K^{248}XP^{221}A^{242}F^{264}XL^{421}$	17/17 (I) 15/15 (D)	0/8 (I) 0/12 (D)	0/9 (I) 0/11 (D)	0/10 (I) 0/13 (D)	0/14 (I) 0/14/ (D)
PRG[I/L/V]F	$P^{168}P^{172}R^{175}G^{502}L^{499}F^{505}$	0/17 (I) 0/15 (D)	1/8 (I) 2/12(D)	2/9 (I) 0/11 (D)	0/10 (I) 0/13 (D)	1/14 (I) 0/14 (D)
SPI*LY	$S^{262}P^{261}I^{260}XL^{269}Y^{267}$	0/17 (I) 0/15 (D)	2/8 (I) 6/12 (D)	2/9 (I) 5/11 (D)	0/10 (I) 0/13 (D)	5/14 (I) 3/14 (D)
DRGLF	$D^{394}R^{393}G^{255}L^{524}F^{526}$	0/17 (I) 0/15 (D)	0/8 (I) 2/12 (D)	1/9 (I) 1/11 (D)	2/10 (I) 2/13 (D)	0/14 (I) 1/14 (D)
PYTL[D/S]K	$P^{221}Y^{267}T^{238}L^{524}XK^{379}$	0/17 (I) 0/15 (D)	0/8 (I) 0/12 (D)	0/9 (I) 0/11 (D)	5/10 (I) 6/13 (D)	0/14 (I) 0/14 (D)
Total		17/17 (I) 15/15 (D)	3/8 (I) 10/12 (D)	5/9 (I) 6/11 (D)	7/10 (I) 8/13 (D)	6/14 (I) 4/14 (D)

(a) Amino acids are indicated with their one-letter code. * and X represents any given amino acid and indicate that no single amino acid was found to match on the basis of the eluted peptide sequence (*) and no specific amino acid residue was therefore to be assigned.

Figures


A
Patients
(Sequences eluted
with intact Ara h 1)
B
Patients
(Sequences eluted
with digested Ara h 1)
C
BN Rats
(Sequences eluted
with intact Ara h 1)
D
BN Rats
(Sequences eluted
with digested Ara h 1)
E
BN Rats
(Dosed with intact Ara h 1)
F
BN Rats
(Dosed with digested Ara h 1)
Frequency (%)
0
5
10
15
20
A F G I L M P V W
C N Q S T Y
D E H K R
Hydrophobic
Polar
Charged

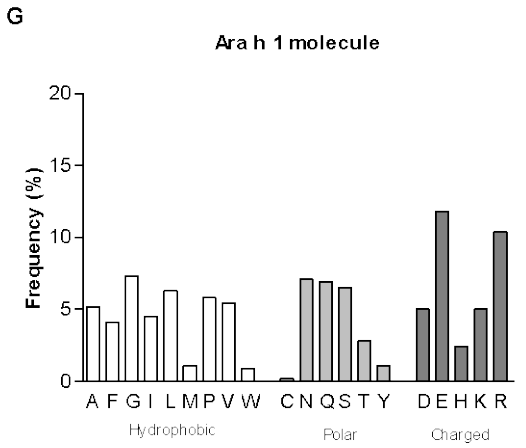

G
Ara h 1 molecule
Frequency (%)
20
15
10
5
0
A F G I L M P V W C N Q S T Y D E H K R
Hydrophobic
Polar
Charged

Fig. 1.

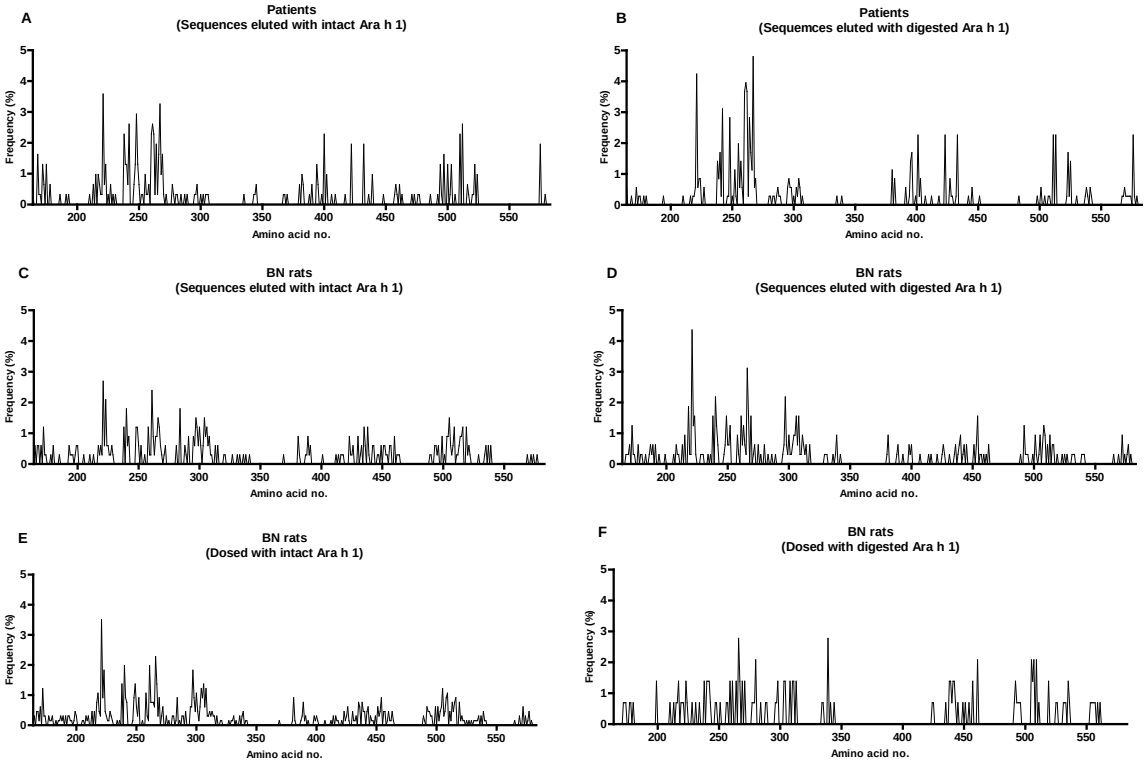

A
Patients
(Sequences eluted with intact Ara h 1)
B
Patients
(Sequemces eluted with digested Ara h 1)
C
BN rats
(Sequences eluted with intact Ara h 1)
D
BN rats
(Sequences eluted with digested Ara h 1)
E
BN rats
(Dosed with intact Ara h 1)
F
BN rats
(Dosed with digested Ara h 1)
Frequency (%)
Amino acid no.

Fig. 2.

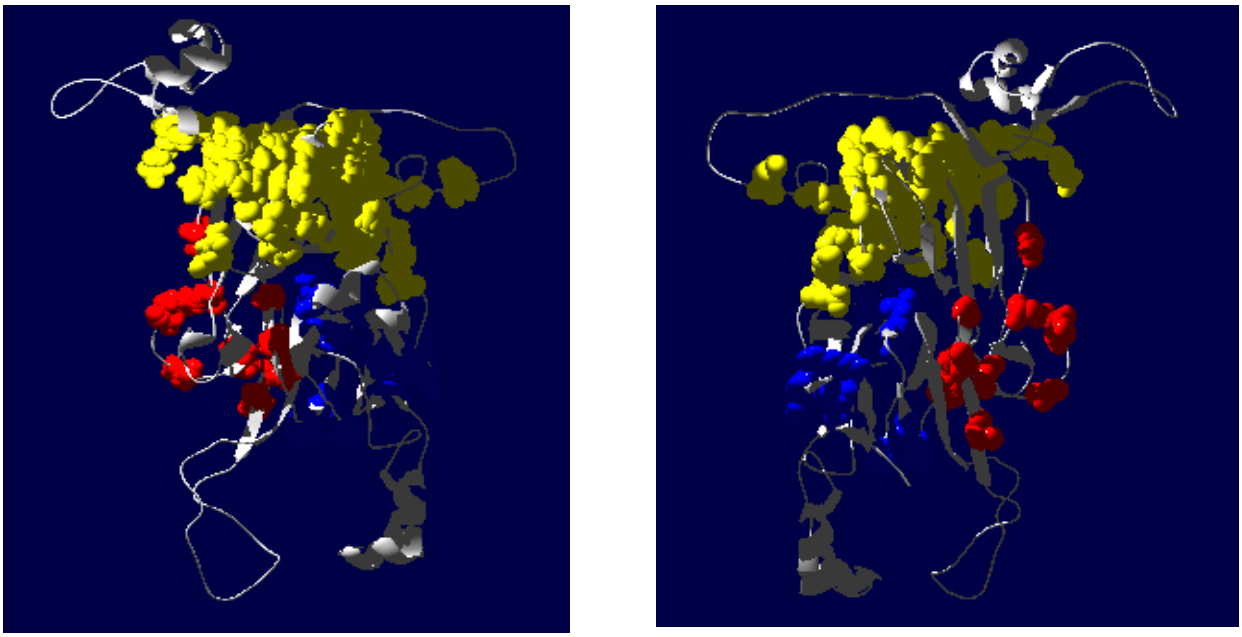


Fig. 3.

Supplementary data

Table A.1: IgE-binding epitope mimics, based on sera from five peanut allergic patients

Eluted with intact Ara h 1		Eluted with digested Ara h 1	
Amino acid sequence of the peptide eluted	Best fitting heptameric epitope sequence	Amino acid sequence of the peptide eluted	Best fitting heptameric epitope sequence
Patient no. 2205			
KAPAFDL (4)	$K^{431}XP^{512}A^{514}F^{577}XL^{399}$ $K^{248}XP^{221}A^{242}F^{264}D^{227}L^{421}$	KAPAFDL (3)	$K^{431}XP^{512}A^{514}F^{577}XL^{399}$ $K^{248}XP^{221}A^{242}F^{264}D^{227}L^{421}$
KAPAFNL	$K^{431}XP^{512}A^{514}F^{577}N^{401}L^{399}$ $K^{248}XP^{221}A^{242}F^{264}XL^{421}$	KAPAFNL	$K^{431}XP^{512}A^{514}F^{577}N^{401}L^{399}$ $K^{248}XP^{221}A^{242}F^{264}XL^{421}$
KLIAFDL (4)	$K^{431}XP^{512}A^{514}F^{577}XL^{399}$ $K^{248}XP^{221}A^{242}F^{264}D^{227}L^{421}$	KEPAFML	$K^{431}XP^{512}A^{514}F^{577}XL^{399}$ $K^{248}XP^{221}A^{242}F^{264}XL^{421}$
KLPAFML (2)	$K^{431}XP^{512}A^{514}F^{577}XL^{399}$ $K^{248}XP^{221}A^{242}F^{264}XL^{421}$	KLPAFML (2)	$K^{431}XP^{512}A^{514}F^{577}XL^{399}$ $K^{248}XP^{221}A^{242}F^{264}XL^{421}$
KQPAFNL (5)	$K^{431}XP^{512}A^{514}F^{577}N^{401}L^{399}$ $K^{248}XP^{221}A^{242}F^{264}XL^{421}$	KLPAFQL	$K^{431}XP^{512}A^{514}F^{577}XL^{399}$ $K^{248}XP^{221}A^{242}F^{264}XL^{421}$
KSPAFNL	$K^{431}XP^{512}A^{514}F^{577}N^{401}L^{399}$ $K^{248}XP^{221}A^{242}F^{264}XL^{421}$	KQPAFNL (2)	$K^{431}XP^{512}A^{514}F^{577}N^{401}L^{399}$ $K^{248}XP^{221}A^{242}F^{264}XL^{421}$
		KSPAFDL (3)	$K^{431}XP^{512}A^{514}F^{577}XL^{399}$ $K^{248}XP^{221}A^{242}F^{264}D^{227}L^{421}$
		KTAAFNL (2)	$K^{431}XP^{512}A^{514}F^{577}N^{401}L^{399}$ $K^{248}XP^{221}A^{242}F^{264}XL^{421}$
Patient no. 2208			
ASPKSLL	$A^{335}S^{300}P^{297}K^{222}S^{266}L^{220}L^{218}$	AKPASWA	$A^{224}K^{222}P^{221}A^{298}S^{304}XA^{335}$
FHTARPW	$F^{295}H^{223}S^{266}A^{242}R^{247}P^{389}X$	DSPRGVF	$D^{194}S^{168}P^{172}R^{175}G^{502}V^{449}F^{505}$
FSPIVLY	$XS^{262}P^{261}I^{260}V^{239}L^{269}Y^{267}$	MSDRGIF (2)	$XXD^{394}R^{393}G^{255}L^{524}F^{526}$
SPIMDYF	$S^{262}P^{261}I^{260}M^{284}D^{225}Y^{267}F^{296}$	SPIINYY	$S^{262}P^{261}I^{260}I^{265}XY^{267}X$
SPPRGIF	$S^{164}P^{168}P^{172}R^{175}G^{502}L^{499}F^{505}$	SPIISHY (2)	$S^{262}P^{261}I^{260}I^{265}S^{266}H^{223}Y^{267}$
TSRADTL	$S^{519}S^{518}A^{514}E^{458}T^{496}L^{449}$	SPILAHY (2)	$S^{262}P^{261}I^{260}L^{258}A^{257}H^{256}Y^{267}$
TTSVRNT	$T^{217}T^{238}S^{249}V^{241}R^{247}N^{246}S^{266}$	SPITLYY	$S^{262}P^{261}I^{260}T^{240}L^{269}Y^{267}X$
VTLAPLR	$V^{400}T^{496}L^{457}A^{514}P^{438}L^{435}R^{581}$ $V^{239}T^{238}L^{269}A^{213}P^{215}L^{277}R^{278}$	VPPRGLF	$S^{164}P^{168}P^{172}R^{175}G^{502}L^{499}F^{505}$
		YDVSSLP	$Y^{180}D^{302}XS^{304}S^{305}L^{307}P^{297}$
Patient no. 2209			
ATETSYR	$A^{242}T^{240}E^{382}T^{238}S^{249}N^{246}R^{247}$	DLTWAPK	$D^{253}L^{252}T^{240}XA^{242}P^{221}K^{222}$ $D^{394}L^{425}T^{425}XA^{509}P^{508}K^{443}$
HAPRGVF	$XP^{168}P^{172}R^{175}G^{502}V^{449}F^{505}$		
LPPRGLF	$XP^{168}P^{172}R^{175}G^{502}L^{499}F^{505}$	FSPIIAF	$XS^{262}P^{261}I^{260}I^{265}A^{257}F^{296}$
MSDRGIF	$XXD^{394}R^{393}G^{255}L^{524}F^{526}$	GLAGYPP	$G^{525}L^{425}A^{257}G^{255}L^{392}P^{389}P^{416}$
SIPYPAP	$S^{173}I^{447}P^{508}Y^{170}P^{168}F^{193}P^{289}$	LSPIIVY	$XS^{262}P^{261}I^{260}I^{265}V^{239}Y^{267}$
SNATWVP	$S^{518}N^{456}A^{497}T^{496}XV^{460}P^{512}$	MSDRGIF	$XXD^{394}R^{393}G^{255}L^{524}F^{526}$
SPIINYY	$S^{262}P^{261}I^{260}I^{265}XY^{267}X$	NLVLFSY	$N^{216}L^{218}V^{381}L^{168}F^{296}S^{266}Y^{267}$
STIMSSR	$S^{266}T^{240}F^{268}S^{381}S^{249}R^{247}$	QWAFAIP (2)	$Q^{303}A^{176}F^{264}A^{224}I^{260}P^{261}$ $Q^{339}XA^{224}F^{295}A^{242}I^{265}P^{297}$
WSPIVHP	$XS^{262}P^{261}I^{260}V^{239}H^{223}X$		
		SPILAHY	$S^{262}P^{261}I^{260}L^{258}A^{257}H^{256}Y^{267}$
		SPIVLYF	$S^{262}P^{261}I^{260}V^{239}L^{269}Y^{267}F^{296}$
		SPIVNNY	$S^{262}P^{261}I^{260}V^{239}XXY^{267}$

Table A.1: IgE-binding epitope mimics, based on sera from five peanut allergic patients. Continued…

Eluted with intact Ara h 1		Eluted with digested Ara h 1	
Amino acid sequence of the peptide eluted	**Best fitting heptameric epitope sequence**	**Amino acid sequence of the peptide eluted**	**Best fitting heptameric epitope sequence**
Patient no. 2304			
AKQTDTM	$A^{280}K^{281}Q^{210}T^{178}D^{302}S^{304}S^{305}$	ADMRGLF	$XD^{394}XR^{393}G^{255}L^{524}F^{526}$
DISRGLF	$D^{394}XXR^{393}G^{255}L^{524}F^{526}$	AKGTDNW	$A^{539}K^{570}G^{540}S^{575}D^{543}N^{544}X$
EWSRGIF	$E^{408}XXR^{393}G^{255}L^{524}F^{526}$	AKQTDTM (2)	$A^{280}K^{281}Q^{210}T^{178}D^{302}S^{304}S^{305}$
IPYTLDK (3)	$I^{268}P^{221}Y^{267}T^{238}L^{524}S^{381}K^{379}$	GSAFSAF	$G^{309}S^{304}A^{517}F^{264}S^{266}A^{224}F^{292}$ $G^{433}S^{299}A^{298}F^{295}S^{579}A^{580}F^{264}$
LHRPLHP	$L^{269}H^{272}R^{345}P^{215}L^{218}XP^{221}$ $L^{523}H^{522}R^{405}P^{397}L^{399}XP^{438}$	GVYSLSK	$XXY^{267}T^{238}L^{524}S^{381}K^{379}$
TPYTLDK (2)	$T^{240}P^{221}Y^{267}T^{238}L^{524}XK^{379}$	IPYTLDK	$I^{268}P^{221}Y^{267}T^{238}L^{524}S^{381}K^{379}$
VTGPAKT	$V^{219}T^{217}G^{343}P^{215}A^{213}R^{186}T^{178}$	LPYTLSK (3)	$L^{220}P^{221}Y^{268}T^{238}L^{524}S^{381}K^{379}$
		SFPARFY	$S^{300}F^{295}P^{221}A^{242}R^{247}F^{250}Y^{267}$
		TPYTLDK	$T^{240}P^{221}Y^{267}T^{238}L^{524}XK^{379}$
		WPDRGIF	$XXD^{394}R^{393}G^{255}L^{524}F^{526}$
Patient no. 2305			
FSPIVLY (2)	$XS^{262}P^{261}I^{260}V^{239}L^{269}Y^{267}$	ACCRSIP	$A^{257}G^{255}C^{427}R^{405}S^{400}I^{430}P^{397}$
KSIYHQW	$K^{248}S^{249}I^{268}Y^{267}H^{223}N^{287}X$	AVESNDK	$A^{580}V^{578}E^{574}S^{575}N^{481}D^{541}K^{570}$
LPSRGLF	$XP^{168}P^{172}R^{175}G^{502}L^{499}F^{505}$	FPPKPKL	$F^{440}P^{512}P^{508}K^{443}E^{531}K^{410}L^{425}$
LTDLTQK	$L^{269}T^{238}D^{253}L^{277}T^{217}Q^{344}R^{345}$	KNVYHQT (2)	$K^{248}N^{246}V^{241}Y^{267}H^{223}N^{287}T^{288}$
QSLTRLP	$Q^{191}S^{273}L^{499}T^{496}R^{494}L^{457}P^{438}$	LINQALK	$L^{568}I^{569}N^{571}Q^{572}A^{539}L^{538}K^{542}$
SPILAHY	$S^{262}P^{261}I^{260}L^{258}A^{257}H^{223}Y^{267}$	MSDRGIF	$XXD^{394}R^{393}G^{255}L^{524}F^{526}$
SPITEFY (2)	$S^{262}P^{261}I^{260}T^{240}E^{382}F^{250}Y^{267}$	SPIISHY	$S^{262}P^{261}I^{260}I^{265}S^{266}H^{223}Y^{267}$
TFKLHPI	$T^{238}F^{250}K^{248}V^{241}H^{223}P^{221}I^{229}$	SPITLYY	$S^{262}P^{261}I^{260}T^{240}L^{269}Y^{267}X$
TPPRVHL	$T^{387}P^{389}P^{416}R^{393}V^{366}H^{367}L^{369}$	SPITTYY	$S^{262}P^{261}I^{260}T^{240}T^{238}Y^{267}X$
VLPGSKS	$V^{231}L^{258}P^{261}G^{263}S^{266}K^{248}S^{381}$	TPFSNSP	$T^{240}P^{221}F^{264}S^{262}N^{287}S^{283}P^{289}$
WESRGVF	$W^{476}E^{475}S^{488}R^{472}G^{470}V^{462}F^{169}$	VMPGSKP (3)	$V^{286}M^{284}P^{261}G^{263}S^{266}K^{248}P^{389}$
YPPKPLH	$Y^{306}P^{297}P^{221}K^{222}XL^{269}Y^{267}$		

Table A.2: IgE-binding epitope mimics, based on sera from five Brown Norway rats.

Eluted with intact Ara h 1		Eluted with digested Ara h 1	
Amino acid sequence of the peptide eluted	**Best fitting heptameric epitope sequence**	**Amino acid sequence of the peptide eluted**	**Best fitting heptameric epitope sequence**
Animal nr. 19; Rat immunised with digested Ara h 1			
AQSQFNS (5)	$A^{298}Q^{303}S^{304}Q^{308}F^{310}N^{313}S^{311}$	APHRHPH	$A^{539}P^{438}H^{439}R^{535}H^{534}P^{522}H^{511}$
AYTLPSR	$A^{461}Y^{495}T^{496}L^{457}P^{172}S^{173}R^{174}$	APKAAPL	$A^{509}P^{508}K^{443}A^{510}A^{461}P^{512}L^{538}$
HFSKVPR	$H^{223}F^{264}S^{264}K^{248}V^{241}P^{261}R^{259}$	AQLAPET	$A^{280}Q^{234}L^{277}A^{213}P^{215}E^{341}T^{217}$
SNAARAY	$S^{442}N^{441}A^{510}A^{461}R^{494}A^{497}Y^{495}$	AQSQFNS (2)	$A^{298}Q^{303}S^{304}Q^{308}F^{310}N^{313}S^{311}$
YHPFLQV	$Y^{267}H^{223}P^{297}F^{296}L^{220}Q^{338}V^{219}$	GGQFGPP	$G^{562}G^{560}Q^{564}F^{558}A^{557}P^{559}L^{556}$
YSTQVRP (2)	$Y^{267}S^{266}T^{240}N^{243}V^{241}R^{247}A^{242}$	IHLPPAL	$I^{536}H^{439}L^{538}P^{438}P^{512}A^{514}L^{435}$
		NQTRTTH	$N^{216}Q^{344}T^{217}R^{271}T^{238}T^{240}X$ $N^{334}Q^{339}T^{217}R^{271}T^{238}T^{240}X$
		QPMINML	$Q^{199}P^{508}M^{445}I^{528}N^{529}M^{524}L^{525}$
		QPPTANA	$Q^{199}P^{508}P^{512}S^{442}A^{509}N^{441}A^{510}$
		TPMAWSQ	$T^{288}P^{289}M^{284}P^{261}F^{264}S^{266}X$
		VHNTRLL	$V^{449}H^{522}N^{451}T^{454}R^{498}L^{499}X$ $V^{231}H^{256}N^{251}T^{238}R^{271}L^{269}X$
Animal nr. 49; Rat immunised with intact Ara h 1			
AYPLRAH	$A^{298}Y^{306}P^{297}L^{307}R^{327}A^{321}N^{320}$	GPPPLPK	$G^{540}P^{438}P^{512}P^{508}I^{508}A^{505}K^{443}$
GLTPSKN	$G^{255}L^{252}T^{240}P^{221}S^{266}K^{248}N^{388}$	HHMHTTR	$H^{511}H^{439}M^{436}N^{516}S^{519}T^{454}R^{498}$
GPPPLPK	$G^{540}P^{438}P^{512}P^{508}I^{505}A^{509}K^{443}$	IAMKPLA	$I^{528}A^{530}M^{445}K^{443}P^{508}F^{390}A^{514}$
HHLHQTN	$H^{511}H^{439}L^{538}K^{542}Q^{572}S^{575}N^{401}$	LPSLPRI	$L^{258}P^{221}S^{266}I^{265}P^{261}R^{259}I^{229}$
LPLPVVR	$L^{435}P^{438}L^{538}P^{512}V^{460}V^{462}R^{493}$	LQSKTLH	$L^{568}Q^{572}S^{575}K^{542}N^{544}I^{536}H^{534}$
NLANKMA	$N^{451}L^{524}A^{280}N^{228}K^{281}M^{284}P^{261}$	NTSNPYT	$N^{451}T^{454}S^{519}N^{516}P^{582}A^{580}S^{579}$
SLYKPHP	$S^{381}L^{269}Y^{267}K^{222}P^{221}H^{223}P^{261}$	QATFSHS	$Q^{339}A^{213}T^{217}I^{268}S^{266}H^{223}S^{262}$
SSYSQLN	$S^{304}S^{305}Y^{306}S^{300}Q^{308}L^{315}N^{313}$	STFLPHP	$S^{249}T^{238}F^{250}L^{258}P^{261}H^{223}P^{221}$
YGTHKSP	$Y^{280}G^{285}T^{217}H^{272}R^{271}T^{238}T^{240}$	TASGLYS (4)	$T^{314}A^{317}S^{311}G^{309}L^{307}Y^{306}S^{305}$
YSIPKSS	$Y^{267}S^{266}I^{260}P^{389}K^{248}S^{249}S^{291}$	VPPQLSR	$V^{219}P^{221}P^{297}Q^{308}L^{331}S^{304}R^{301}$
VTAPFRV	$V^{460}T^{496}A^{497}P^{172}P^{168}R^{165}X$	VTAPFRV	$V^{460}T^{496}A^{497}P^{172}P^{167}R^{165}X$
		VYPMAMS	$V^{492}Y^{495}P^{172}M^{507}A^{510}M^{445}S^{442}$
		YHGSVSL	$Y^{306}H^{223}G^{263}S^{266}V^{241}S^{249}L^{252}$
Animal nr. 52; Rat immunised with intact Ara h 1			
AMPPLPP	$A^{510}M^{507}P^{172}R^{498}I^{515}P^{438}P^{512}$		
HPWAPMQ	$H^{223}P^{297}F^{295}A^{266}P^{172}M^{284}Q^{291}$		
KPPNLPN	$K^{414}P^{416}P^{389}N^{391}L^{258}P^{261}N^{228}$		
KWLPTPL	$K^{542}F^{537}L^{538}P^{512}S^{442}P^{508}I^{506}$		
SASWQES	$S^{299}A^{298}S^{305}F^{296}Q^{303}E^{333}X$		
SLTSWAT	$S^{381}L^{269}T^{240}S^{266}F^{264}A^{242}S^{249}T^{238}$		
STFLPHP (7)	$S^{249}T^{238}F^{250}L^{258}P^{261}H^{223}P^{221}$		
TQMSKHL	$T^{288}Q^{291}M^{284}S^{262}A^{224}H^{223}I^{265}$		
VIAKTRL	$V^{219}I^{265}A^{242}K^{248}T^{240}R^{259}L^{258}$		

Table A.2: IgE-binding epitope mimics, based on sera from five Brown Norway rats. Continued…

Eluted with intact Ara h 1		Eluted with digested Ara h 1	
Amino acid sequence of the peptide eluted	**Best fitting heptameric epitope sequence**	**Amino acid sequence of the peptide eluted**	**Best fitting heptameric epitope sequence**
Animal nr. 53; Rat immunised with intact Ara h 1			
ALGASHG	$A^{514}L^{435}G^{433}A^{517}S^{519}H^{522}G^{453}$	AKTSSNV	$A^{242}K^{248}S^{249}T^{240}S^{381}N^{251}L^{252}$
AQSQFNS (4)	$A^{298}Q^{303}S^{304}Q^{308}F^{310}N^{313}S^{311}$	APLPKLM	$A^{213}P^{215}L^{218}P^{221}K^{222}L^{230}S^{266}$
FHDTPQS	$F^{295}H^{223}D^{225}T^{288}P^{289}Q^{291}M^{284}$	AQSQFNS (3)	$A^{298}Q^{303}S^{304}Q^{308}F^{310}N^{313}S^{311}$
HISLGRI	$H^{522}I^{530}S^{518}L^{499}G^{502}R^{175}V^{504}$	DQWHRAP	$D^{194}Q^{199}P^{512}H^{511}R^{463}A^{461}P^{508}$
LGTYGHY	$L^{315}G^{309}S^{304}Y^{306}F^{295}H^{223}Y^{267}$	FGFPTTS	$F^{264}G^{263}F^{295}P^{221}T^{240}T^{238}S^{381}$
NPASSHM	$Q^{583}P^{582}A^{580}S^{518}S^{519}H^{522}I^{430}$	FPTPRVA	$F^{296}P^{297}S^{299}P^{221}R^{247}V^{241}A^{252}$
NYLSLLH	$N^{167}Y^{170}I^{506}S^{173}L^{499}L^{457}N^{456}$	HQPQQLF	$H^{272}Q^{275}P^{215}Q^{234}Q^{236}L^{252}F^{250}$
QIPSGTP	$Q^{417}I^{390}P^{389}T^{387}K^{248}T^{240}P^{221}$	ISVNIQA	$I^{187}S^{177}V^{189}Q^{191}I^{208}Q^{210}A^{280}$
QLSAPPP	$Q^{308}L^{331}S^{304}A^{298}P^{297}P^{221}A^{242}$	IVQHTVP	$I^{187}V^{189}Q^{191}H^{206}S^{283}V^{286}P^{289}$
SASLMLQ	$S^{518}A^{217}S^{577}L^{435}M^{436}L^{457}N^{456}$	KPWLPQH	$K^{222}P^{221}Y^{267}L^{218}P^{215}Q^{275}H^{272}$
TMSKDST	$T^{426}M^{423}M^{284}K^{281}D^{227}S^{262}X$	NAHHTYP	$N^{456}A^{514}H^{439}H^{511}T^{496}Y^{495}P^{172}$
YPPMTQV	$Y^{170}P^{168}P^{172}M^{507}P^{508}Q^{199}F^{200}$	NHTTLKA	$N^{451}H^{522}T^{454}S^{518}L^{435}K^{431}A^{580}$
YTQGWNL	$Y^{180}T^{178}Q^{210}G^{502}V^{449}N^{451}L^{524}$	NPLPSQL	$N^{388}P^{389}L^{258}P^{261}S^{262}N^{228}L^{421}$
		QPVPPRL	$Q^{339}P^{261}V^{241}P^{297}P^{221}K^{222}L^{230}$
		SDRFPPK	$S^{575}D^{543}R^{535}F^{440}P^{512}P^{508}K^{443}$
		SDYHRVM	$S^{304}D^{302}Y^{180}R^{179}R^{188}V^{209}S^{177}$
		SLDTCLR	$S^{400}L^{399}D^{498}T^{426}C^{427}L^{425}R^{393}$
		SPSPQRS	$S^{305}P^{297}S^{266}P^{221}Q^{308}R^{301}S^{311}$
		TGGVWSK	$T^{454}G^{453}G^{502}V^{504}F^{505}S^{173}R^{174}$
		YNPAVIA	$Y^{170}N^{167}P^{172}A^{497}V^{504}I^{208}A^{280}$
Animal nr. 54; Rat immunised with intact Ara h 1			
AETVESC	$A^{213}E^{341}T^{217}V^{219}E^{338}S^{300}S^{305}$	FTDTSWM	$F^{407}T^{426}D^{398}S^{400}S^{575}F^{577}M^{436}$
DAFTRGT	$D^{225}A^{224}F^{296}S^{304}R^{301}G^{336}S^{300}$	HGWPVPK	$H^{223}G^{263}F^{264}P^{261}V^{241}P^{389}K^{248}$
GMTMATP (2)	$G^{525}M^{424}T^{426}M^{423}M^{284}S^{262}P^{261}$		$H^{223}G^{263}F^{264}P^{261}V^{241}P^{416}K^{414}$
GPPPLPK	$G^{540}P^{438}P^{512}P^{508}I^{505}A^{509}K^{443}$	ITLQRTF	$I^{268}T^{217}L^{218}Q^{339}R^{186}T^{178}F^{176}$
GVGVPQR (2)	$G^{502}V^{449}G^{525}V^{446}P^{508}Q^{199}R^{196}$		$I^{268}T^{217}L^{277}Q^{236}R^{271}T^{238}F^{250}$
IPSLPMR	$I^{173}P^{221}S^{266}L^{307}P^{297}S^{304}R^{301}$	LHGRYFP	$L^{459}H^{256}G^{255}R^{393}F^{407}T^{400}P^{397}$
LPVPIGY	$L^{220}P^{221}V^{241}P^{261}I^{265}G^{263}Y^{306}$	LTSPLRL	$L^{252}T^{240}S^{266}P^{221}L^{218}R^{271}L^{277}$
MHTQDLM	$M^{507}H^{511}S^{442}N^{532}D^{412}L^{418}M^{423}$	QGSQYTQ	$Q^{339}G^{336}S^{300}Q^{303}Y^{306}S^{305}Q^{308}$
MLNATSK	$M^{436}L^{435}N^{516}A^{517}T^{454}S^{519}K^{452}$	TGSSNLY	$T^{454}G^{453}S^{519}S^{518}N^{516}L^{457}Y^{495}$
QDWNFKK	$Q^{195}D^{194}F^{193}N^{205}F^{200}K^{198}K^{464}$		$T^{238}G^{380}S^{381}S^{249}N^{251}L^{269}Y^{267}$
SGEIHFK	$S^{519}G^{433}E^{432}I^{430}H^{522}L^{523}K^{452}$	TLLTTSP	$T^{217}L^{218}L^{269}T^{238}T^{240}S^{266}P^{221}$
STFLPHP	$S^{249}T^{238}F^{250}L^{258}P^{261}H^{223}P^{221}$	TMTTPQQ	$T^{217}L^{218}T^{238}T^{240}P^{221}Q^{339}Q^{183}$
VIAKTRL (2)	$V^{219}I^{265}A^{242}K^{248}T^{240}R^{259}L^{258}$	TTAPGKP	$T^{240}S^{266}A^{224}P^{261}G^{263}K^{222}P^{221}$
VPFKPIR (2)	$V^{241}P^{261}F^{264}K^{222}P^{221}I^{268}R^{271}$	TTHYLHA	$T^{240}S^{266}H^{223}Y^{306}L^{307}R^{312}A^{317}$
VTAPFRV (3)	$V^{460}T^{496}A^{497}P^{172}P^{167}R^{165}X$	WPELYPV	$F^{296}P^{297}E^{338}L^{218}Y^{267}P^{221}V^{241}$
YPTRELS	$Y^{267}P^{221}T^{240}R^{247}E^{382}L^{252}S^{381}$		$F^{440}P^{438}E^{458}L^{457}Y^{495}P^{172}V^{504}$
		YSYPGLT	$Y^{267}S^{266}Y^{307}P^{306}A^{298}L^{330}T^{314}$

PAPER 4

(Submitted)

Co-administration of intact and digested *β*-lactoglobulin may induce tolerance

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Abstract

Background: It is generally believed that protein hydrolysis in the gastrointestinal tract decreases the allergenicity of food allergens. It remains however unsolved, if specific properties of digestion products determine whether a sensitisation or tolerogenic immune response will develop. We sought to examine the sensitising capacity of the cow's milk allergen *β*-lactoglobulin (BLG) and digestion products hereof in a Brown Norway (BN) rat model of food allergy.
Methods: Intact BLG was digested in an *in vitro* model simulating the gastro-duodenal digestion process and subsequently fractionated by gel permeation chromatography. BN rats were dosed with either PBS, 200 µg of intact BLG, 30 µg of intact BLG, 200 µg of partially digested BLG, 200 µg of digested BLG, or with 200 µg of a fraction of large complexes or a fraction of small complexes. Sera from BN rats were analysed for specific antibodies and avidity was measured.
Results: BLG partly resisted the digestion process. However, the BLG molecules that did not survive the digestion process were rapidly broken down to peptides of sizes less than M_r 4,500. Specific antibody responses revealed that both 200 and 30 µg of intact BLG had immunogenic as well as sensitising capacity, while digested BLG could not induce any specific antibodies. Most importantly, while intact BLG showed a significant sensitising capacity when administered alone, this sensitising capacity was significantly reduced when co-administered with digested BLG.
Conclusions: Co-immunisation with intact and digested BLG reduces the sensitising capacity of intact BLG, probably by tolerogenic mechanisms introduced by digestion products.

Introduction

Cow's milk allergy (CMA) is a general health problem, mostly affecting young children. The prevalence of IgE-mediated CMA is estimated to be around 2.5% for infants [1], whereupon 80-85% of these children outgrow their allergy [2], resulting in less than 1% of adults suffering from CMA [3]. Cow's milk contains several proteins able to induce allergic responses. The major cow's milk allergen BLG, also designated Bos d 5, is the most abundant protein in the milk whey fraction. It belongs to the lipocalin allergen family and contains two disulphide bonds and one free cysteine group [3, 4]. BLG consists of 162 amino acid residues, corresponding to a M_r of 18,300. Two isoforms of BLG exist and in physiological surroundings, these appear in dimer forms, which stabilises the structure of this globular protein [3, 4]. Numerous studies have been performed with the aim to identify BLG-specific epitopes. They indicate that many conformational as well as many linear epitopes exist, covering most of the molecule [5-7].
It is generally believed that dietary proteins must survive the gastrointestinal digestion process, as intact proteins or as large peptide fragments, to be absorbed and recognised by the intestinal mucosal immune system [8, 9]. This believe may partly be based on a study by Astwood et al. [9] showing that food allergens were more resistant to simulated gastric fluid compared to proteins of no proven allergenicity. Later studies have though shown that resistance to digestion is not an absolute characteristic of food allergens [10-12]. BLG is found to be a very stable protein, resisting both simulated gastric as well as gastro-duodenal digestion processes. The resistance of BLG to digestion coupled with its absence from human breast milk has been proposed to contribute to the major allergenicity of BLG [3, 4].

It is generally accepted that degradation of BLG reduces its allergenicity, but a few studies have shown digestion products of BLG to inhere similar IgE reactivity as the intact protein [13] or even greater IgE

reactivity than the intact BLG [14]. Such results indicate that the native BLG structure is not a necessity for the allergenicity of BLG and that linear IgE epitopes could be of importance for the reactivity of BLG, and further could suggest that humans may be sensitised to the denatured and digested BLG in addition to the intact protein.

Hypoallergenic milk formulas based on hydrolysis products of whey or caseins are available for infants with CMA, and are divided into extensively hydrolysed formulas (eHF) and partially hydrolysed formulas (pHF), according to the degree of protein hydrolysis and the molecular weight of the present peptides. The purpose of eHF is to degrade proteins to such a degree that all potential allergenicity is lost, however, the likely concomitant loss of immunogenicity prevent the immune system from developing tolerance to the milk proteins. In contrast, pHF is designed to minimise the sensitising capacity, though at the same time containing peptides, with a size big enough to be recognised by the intestinal immune system for induction of oral tolerance [15]. While eHFs in general have been estimated to cause allergic reactions in approximately 5% of CMA infants, pHF is estimated to cause allergic reactions in between 33% and 50% of CMA infants [16]. While eHFs are considered efficient in treatment of infants already sensitised to cow's milk protein, pHFs are on the other hand suggested to be useful for the prevention of cow's milk sensitisation [15, 16]. These considerations are in agreement with animal studies, indicating that pHF is able to induce oral tolerance, whereas eHF is not [17-19]. For example, van Esch et al. [19] showed in a mouse model that whey based pHF given by gavage could induce tolerance to whey in contrast to eHF also based on whey, and Fritsche et al. [18] showed in a rat model that oral administration of whey based pHF was able to induce specific oral tolerance to BLG, whereas an eHF equally based on whey was not. Likewise, in the same rat model, moderately hydrolysed soy proteins could induce oral tolerance to intact soy proteins, whereas strongly hydrolysed soy proteins were not able to achieve this [17]. Nevertheless, Crittenden and Bennett [15] emphasise that no clinical studies completely confirms the animal studies and Bahna [16] state that both pHF and eHF can be useful for prevention of CMA.

Fortunately in most people the intestinal immune system recognises the milk proteins as harmless and develops oral tolerance. This immunological active mechanism of unresponsiveness is believed to be achieved through deletion or anergy of the allergen-specific T cells or by the generation of regulatory T cells [20]. The mechanisms of oral tolerance induction versus sensitisation to dietary proteins remain generally unknown, but CMA is believed to form as a result of either failure or breakdown of tolerogenic processes [3].

In the current study we sought to examine the sensitising capacity of intact BLG as well as different well-characterised digestion products hereof in a BN rat model.

Material and Methods

Simulated gastro-duodenal digestion of BLG

A pilot batch of purified BLG was kindly delivered by Arla Food Ingredients (Videbæk, Denmark). The endotoxin content was tested by Lonza endotoxin testing service (Lonza, Verviers, Belgium) to be < 0.01 EU per mg of BLG.

Digestion was performed essentially as described by Bøgh et al. [11], with immobilised enzymes, in order to produce enzyme-free digests suitable for animal sensitisation studies. Addition of surfactant (phosphatidylcholine and bile salts) were omitted in order to avoid adverse effects on the sensitisation.

Briefly, for simulated gastric digestion, pepsin immobilised to agarose (P0609, Sigma, Saint Louis, MO, USA) was washed twice in 1 mM HCl (1000 x *g*, 2 min). Purified BLG (20 mg/mL in Milli Q water (Water drawn

from a Milli Q System equipped with an Organex cartridge from Millipore (Bedford, MA, USA)) was adjusted to pH 2.5 with 6 M HCl and was added to the immobilised pepsin to yield a pepsin activity of approximately 180 U per mg of BLG. The solution was placed in a shaking incubator (200 rpm, 37 °C) for 120 min. Reaction was stopped by adjusting pH to 7 with 6 M NaOH, and immobilised pepsin was removed by centrifugation (1000 x *g*, room temperature (RT), 2 min) followed by filtrating supernatant through a 22 µm filter (Sterile Syringe Filter, CA 0.2 µm, Frisenette Aps, Knebel, Denmark).
For simulated duodenal digestion, $CaCl_2$ and Bis-Tris propane was added to the solution of gastric digest of BLG, to yield a final concentration of 9.2 mM $CaCl_2$ and 24.7 mM of Bis-Tris propane. pH was adjusted to 6.5 with 6 M HCl. Trypsin immobilised to agarose (T1763, Sigma) and chymotrypsin immobilised to agarose (C9134, Sigma) were washed twice in Milli Q water and once in 9.2 mM $CaCl_2$, 24.7 mM Bis-Tris propane and added to ¼ of the gastric digest of BLG to yield an activity corresponding to 34.5 U of soluble trypsin per mg of BLG digest and 0.44 U of soluble chymotrypsin per mg of gastric BLG digest (clarified in activity assays using BAPA as trypsin substrate and SUNA as chymotrypsin substrate). The solution was placed in a shaking incubator (200 rpm, 37 °C) for 15 min. Reaction was stopped by centrifugation (1000 x g, RT, 2 min) followed by filtrating supernatant through a 0.22 µm filter (Syringe Filter). To make all gastric digest undergo simulated duodenal digestion, the duodenal digestion phase was repeated three times and BLG digest from the four runs were pooled. (Reusability of enzymes was confirmed by comparing the digestion product profiles for the four runs by reverse phase high-performance liquid chromatography (RP-HPLC), data not shown).

Fractionation of BLG digests
Fractionation by preparative gel permeation chromatography (GPC) of gastro-duodenal digests of BLG was performed essentially as described in Bøgh et al. [21]. Separated fractions were collected and pooled according to the preparative GPC profile shown in Fig. 1, resulting in four different pools of BLG-digests, in the following designated: partially digested BLG, digested BLG, large complexes and small complexes, where partially digested BLG corresponds to the whole pool of BLG digests, containing intact BLG that resisted the digestion process, and where digested BLG corresponds to the combined large and small complexes and thereby the whole pool of digested BLG without any intact BLG.

RP-HPLC analysis
For analysis of purity and presence of residual intact BLG and for evaluation of chromatography profiles, RP-HPLC was performed as previously described [21].

Matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS)
For analysis of peptide mass distributions in pools of BLG digests MADLI-TOF MS was performed as previously described [21].

Amino acid analysis
For determination of concentrations and for examination of amino acid compositions amino acid analysis was performed by ion-exchange chromatography after hydrolysis in HCl overnight, as described by Barkholt and Jensen [22].

GPC analysis
For analysis of the peptide aggregation profiles in the different pools of BLG digests analytical GPC was performed as previously described [21].

Animals

BN rats were from our in-house breeding colony at the National Food Institute (Technical University of Denmark, Denmark), weaned at three weeks of age and then housed in macrolon cages (two per cage) with light:dark cycle, at 22 ± 1 °C and 55 ± 5% relative humidity. Rats were observed twice daily and clinical signs recorded.

Rats were kept on a diet free of milk for at least three generations to avoid tolerance against BLG, which like in human breast milk is not a constituent of rat's milk. The rat diet was produced in-house and based on rice flour, potato protein and fish meal as protein sources, as previously described [11], with the exception of maize flakes being substituted with rice flour. Diet and acidified water was given *ad libitum*.

Experimental design

For studying the sensitising capacity of intact BLG, partially digested BLG, digested BLG and the two fractions hereof, BN rats, 4-9 weeks of age, were allocated into seven groups. Each group consisted of 12 rats, besides the group immunised with 200 μg of intact BLG, which consisted of 20 rats. Rats were immunised i.p. with either PBS (control), 200 μg of intact BLG, 30 μg of intact BLG, 200 μg of partially digested BLG (which contained 30 μg of intact BLG), 200 μg of digested BLG (without any intact BLG), 200 μg of large complexes or 200 μg of small complexes. Rats were immunised without any use of adjuvant, three times, at day 0, 14 and 28 and sacrificed at day 35 by exsanguination using carbon dioxide inhalation as anesthesia. Blood was collected and converted to sera, which was stored at -20 °C until use for analyses. I.p. sensitisation procedure was chosen for avoidance of *in vivo* digestion of antigens as well as for the small amount of antigens needed for immunisation.

Positive control sera were produced by i.p. immunisation of BN rats three times, at day 0, 14 and 28 with 200 μg of intact BLG in 2% Alhydrogel in PBS as adjuvant. Rats were sacrificed at day 35 and blood collected.

Animal experiments were carried out at the National Food Institute (Technical University of Denmark) facilities under conditions approved by the Danish Animal Experiments Inspectorate and the in-house Animal Welfare Committee.

Enzyme-linked immunosorbent assay (ELISA) for detection of specific IgG1 and IgG2a

For detection of IgG1 and IgG2a antibodies specific for intact BLG, partially digested BLG, digested BLG as well as the two fractions of digested BLG, ELISA was performed. Plates (96 well, microtitre, Maxisorp, Nunc, Roskilde, Denmark) were coated with 100 μL/well of 10 μg/mL antigen solution in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and incubated overnight at 4 °C. Between each step, plates were washed five times in PBS with 0.01% (w:v) Tween 20 (PBS-T). Two-fold serial dilutions of serum (starting at 1:8, v:v) in PBS-T, 50 μL/well, were added and incubated for 1 h at RT. For detection, 50 μL/well of either HRP-labelled mouse-α-rat IgG1 (3060-05, Southern Biotech, Birmingham, AL, USA) diluted 1:20,000 (v:v) in PBS-T or HRP-labelled mouse-α-rat IgG2a (03-9620, Invitrogen, Camarillo, CA, USA) diluted 1:2,000 (v:v) in PBS-T was added to each well and incubated for 1 h at RT. Reaction was visualised by adding 100 μL/well of 3,3',5,5-tetramethylbenzidine (TMB)-one substrate (Kem-En-Tec, Taastrup, Denmark) for approximately 12 min and stopped with 100 μL/well of 0.2 M H_2SO_4. Absorbance was measured at 450 nm with a reference wavelength of 630 nm, using a microtitre reader (Gen5, BioTek Instruments, Winooski, VT, USA). Antibody titres were expressed as the Log_2 titre values and defined as the interpolated dilution of the given serum sample leading to the mean absorbance for the negative control serum + 3 SD, correlating to absorbance values of less than OD 0.1 for both IgG1 and IgG2a.

Antibody-capture ELISA for detection of specific IgE

For detection of BLG-specific IgE antibody-capture ELISA was performed, where plates (96 well, Maxisorp, Nunc) were coated with 100 µL/well of 0.5 µg/mL mouse-α-rat IgE (HDMAB-123 HybriDomus, Cytotech, Hellebæk, Denmark) in carbonate buffer and incubated overnight at 4 °C. Between each step, plates were washed five times in PBS-T. Plates were blocked for 1 h at 37 °C in 200 µL/well of 3% (w:v) rabbit sera. Two-fold serial dilution of serum (starting at 1:8, v:v) in PBS-T, 50 µL/well, were added and incubated for 1 h at RT. Subsequently, plates were incubated with 50 µL/well of 0.2 µg/mL of digoxigenin-coupled BLG (10:1) in 3% rabbit sera in PBS-T for 1 h at RT. Plates were then incubated with 100 µL/well of HRP-labelled sheep-α-digoxigenin (Anti-Digoxigenin-POD 1 633 716 001, Roche Diagnostics GmbH, Mannheim, Germany) diluted 1:1,000 (v:v) in PBS-T for 1 h at RT. Reaction was visualised by adding 100 µL/well of TMB-one substrate (Kem-En-Tec) for approximately 12 min and stopped with 100 µL/well of 0.2 M H_2SO_4. Absorbance was measured at 450 nm with a reference wavelength of 630 nm, using a microtitre reader (Gen5, BioTek Instruments). IgE antibody titres were expressed as the Log_2 titre values and defined as the interpolated dilution of the given serum sample leading to the mean absorbance for the negative control serum + 3 SD.

ELISA for detection of specific IgA

For detection of BLG-specific IgA plates (96 well, Maxisorp, Nunc) were coated with 100 µL/well of 10 µg/mL intact BLG in carbonate buffer and incubated overnight at 4 °C. Between each step, plates were washed five times in PBS-T. Two-fold serial dilution of serum (starting at 1:8, v:v) in PBS-T, 50 µL/well, were added and incubated for 1 h at RT. For detection, 50 µL/well of HRP-labelled goat-α-rat IgA (STAR 111P, AbD Serotec, Oxford, United Kingdom) diluted 1:5,000 (v:v) in PBS-T was added to each well and incubated for 1 h at RT. Reaction was visualised by adding 100 µL/well of TMB-one substrate (Kem-En-Tec) for approximately 12 min and stopped with 100 µL/well of 0.2 M H_2SO_4. Absorbance was measured at 450 nm with a reference wavelength of 630 nm, using a microtitre reader (Gen5, BioTek Instruments). Antibody titres were expressed as the Log_2 titre values and defined as the interpolated dilution of the given serum sample leading to the mean absorbance for the negative control serum + 3 SD.

Avidity measurements

For measurement of the strength of binding between antigens and IgG1 antibodies a thiocyanate inhibition ELISA based on the method described by El-Khouly et al. [23] was conducted. Plates (96 well, Maxisorp, Nunc) were coated with 100 µL/well of 10 µg/mL antigen solution in carbonate buffer and incubated overnight at 4 °C. Between each step, plates were washed five times in PBS-T. Serum samples were diluted in PBS-T to give an OD between 0.5 and 1 and 50 µL/well were added in six rows of quadruplicates for each serum sample. After incubation for 1 h at RT, 50 µL/well of potassium thiocyanate (KSCN, Sigma, St. Louis, MO, USA) diluted in 1% bovine serum albumin in PBS-T (w:v) was added to the plates in increasing concentrations (0, 0.1, 0.2, 0.5, 1 and 2 M) and incubated for 30 min at RT. For detection, 50 µL/well of HRP-labelled mouse-α-rat IgG1 (3060-05, Southern Biotech) diluted 1:20,000 (v:v) in PBS-T was added to each well and incubated for 1 h at RT. Reaction was visualised by adding 100 µL/well of TMB-one substrate (Kem-En-Tec) for approximately 12 min and stopped with 100 µL/well of 0.2 M H_2SO_4. Absorbance was measured at 450 nm with a reference wavelength of 630 nm, using a microtitre reader (Gen5, BioTek Instruments).

Under the given assay conditions, it was determined that KSCN did not influence the binding of antigen to the plates.

Avidity results of individual serum samples are expressed as the concentration of KSCN required for inhibition of 50% (IC_{50}) of antigen-antibody binding, defined as a 50% reduction of OD, so that the lower the concentration of KSCN needed for 50% inhibition the lower the avidity of antigen-antibody interactions. Calculations were performed as described by El-Khouly et al. [23].

Curve calculations and Statistical analyses

Curve calculations (XY analyses) and statistical calculations were made using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA). Antibody titres were examined for group differences, using the non-parametric One-way ANOVA, Kruskal-Wallis test, followed by Dunn's multiple comparison test for comparison of three or more groups. Curves (KSCN concentration against % reduction in absorbance) used for calculation of IC_{50} were tested for variances by the One-way ANOVA, Bartlett's test for equal variances, followed by Turkey's multiple comparison test. No significant differences between curves appeared, wherefore avidity measures expressed as IC_{50} were examined for group differences, using a parametric One-way ANOVA, followed by the Tukey's multiple comparison test for comparison of three or more groups. Differences between groups of animals were regarded as significant when $P \leq 0.05$. Asterisks indicate a statistically significant difference between the given group and the control group. Asterisks over a horizontal line indicate a statistically significant difference between the two given groups. *=$P \leq 0.05$, **=$P \leq 0.01$, ***$P \leq 0.001$

Results

Characteristics of intact BLG and BLG digests

BLG was digested in an *in vitro* model simulating the gastric as well as duodenal digestion process and subsequently subjected to prep grade GPC for separation of the digestion products by means of fractionation. From the prep grade GPC profile it was decided to make four different pools of BLG digests; partially digested BLG (corresponding to the whole pool of BLG digests), digested BLG (corresponding to all digestion products without any intact BLG), large complexes (corresponding to the fraction of peptides in digested BLG aggregating to the largest complexes) and small complexes (corresponding to the fraction of peptides in digested BLG only aggregating to small complexes or not aggregating at all) (Fig. 1).

From analytical RP-HPLC analyses it was evident that BLG is a relatively resistant protein, being virtually completely resistant to proteolysis with pepsin (Fig. 2B), while being degraded to a greater extent by trypsin and chymotrypsin (Fig. 2C). Intact BLG was eluted at a retention volume of 4.6 mL (Fig 2A), revealing that some residual intact BLG was left after termination of the gastro-duodenal digestion process (Fig. 2C). By calculation of the area under the 220 nm RP-HPLC absorbance curve it was determined that approximately 15% intact BLG survived 2 h of simulated gastric digestion plus 15 min of simulated duodenal digestion.

From RP-HPLC analyses it was evident that removal of residual intact BLG from the digestion products was successful, since no detectable peaks were evident at an elution volume of 4.6 mL corresponding to the elution volume for intact BLG in the chromatography profiles for neither digested BLG nor any of the fractions (Fig. 2A vs. D-F). While the chromatography profiles of the digestion products of partially digested BLG and digested BLG were virtually identical (Fig. 2C-D) it was apparent that chromatography profiles changed when digestion products were separated into fractions (Fig. 2D-F), suggesting that peptide composition varies between the two fractions of large and small complexes and hereby differs from the peptide composition of the whole pool of digested BLG.

A comparison of the total amino acid composition of intact BLG and the different pools of BLG digests, revealed the amino acid distribution to be essentially identical for intact BLG, partially digested BLG and digested BLG (Fig. 3A-C), indicating that no peptide fragments generated in the digestion process were lost in the separation of residual intact BLG from digestion products and that digested BLG contains a peptide composition representative of the intact BLG, with hydrophobic amino acids responsible for approximately 45%, the polar for approximately 16% and the charged for approximately 39% of total amino acid residues. In contrast, the amino acid distribution of the two fractions of digested BLG differs from each other and thereby from that of intact BLG, with most hydrophobic amino acids gathered in the small complexes and most charged amino acid gathered in the large complexes (Fig. 3), indicating that the charged peptides are the ones aggregating to the largest complexes. The amino acid analyses confirms the result of RP-HPLC suggesting that peptide fragments of digested BLG were not distributed equally by the fractionation process and that the peptide profiles of the large complexes and small complexes were not representative of the intact BLG.

For examination of the peptide mass distribution of the three pools of digested BLG without intact protein, MALDI-TOF MS was performed, which demonstrated that the intact BLG which did not escape the digestion process, was digested to small peptide fragments with an apparent M_r of up to 4,500, of which approximately 75% had a M_r between 500 and 2,000 (Fig. 4). As in the whole pool of digested BLG, the peptides in the large complexes were less than M_r 4,500, while in the small complexes the peptides were less than M_r 3,000. This indicates that while the peptides in the digested BLG and large complexes were composed of up to approximately 38 amino acid residues, the peptides in the small complexes were composed of up to approximately 25 amino acid residues.

To analyse if the peptides did aggregate and if so to which degree, analytical GPC was performed. Intact BLG was eluted as a single peak corresponding to a M_r of around 36,000, revealing that BLG existed as a dimer (Fig 5A). A peak corresponding to the dimerised BLG is also evident in the analytical GPC profile of partially digested BLG, and from calculation of the area under the 220 nm curve it was revealed that this peak corresponded to approximately 15% of the total area, (equivalent to the 15% residual intact BLG revealed from the RP-HPLC absorbance curve), together indicating that the dimerised intact BLG did not aggregate with the peptide fragments generated from the digestion process (Fig. 5B). From analyses of the GPC profiles and the area under the 220 nm absorbance curves corresponding to the peptide fragments emerging form the gastro-duodenal digestion, shown in Fig. 5B-E, it was revealed that a substantial part of the peptides indeed did aggregate to larger complexes. The analytical GPC profiles of the peptide fragments in partially digested BLG and digested BLG appeared essentially identical and for both the partially digested BLG and the digested BLG, it was indicated that approximately 43% of the peptide fragments were in aggregated complexes between apparent M_r 28,000 and 4,500 (Fig. 5B-C). This indicates that the fractionation procedure when separating intact BLG from the digestion products did not influence aggregation state of the peptides. However, by separating the digested BLG into two fractions, the aggregation state was disturbed, making the complex-formation of the peptides change (Fig. 5C-E). While approximately 80% of the peptide fragments in the large complexes were aggregated to complexes between M_r 32,500 and 4,500, only approximately 32% of the peptide fragments in the small complexes were aggregated to complexes between M_r 13,250 and 3,000.

Specific antibody-responses

Sera from BN rats immunised with either PBS (control), 200 μg of intact BLG, 30 μg of intact BLG, 200 μg of partially digested BLG (containing 30 μg of intact BLG), 200 μg of digested BLG (without any intact BLG), 200 μg of large complexes or 200 μg of small complexes, were evaluated for specific antibody responses against

intact BLG as well as the different pools of BLG digests. Analyses of specific IgG1 and IgG2a responses, revealed that while both the high (200 μg) and the low (30 μg) amount of intact BLG as well as the partially digested BLG could induce specific IgG antibodies, though only intact BLG alone to a statistically significant degree, neither the digested BLG nor the two fractions hereof could induce any specific IgG response. Specific IgG1 and IgG2a results were very similar, for which reason only the IgG1 results are shown in Fig. 6. The high and the low immunisation dose of intact BLG induced specific IgG1 reaching equal antibody titre levels in the BN rats that reacted similarly with intact BLG and the partially digested BLG. However, only IgG1 from sera raised in BN rats immunised with the high dose of intact BLG was able to react with digested BLG and the large complexes, indicating differences in IgG1 antibody specificities. From Fig. 6 it was evident that while digestion of BLG abolished the antibody inducing capacity of the resulting peptide fragments, both the whole pool of digested BLG and the large complexes retained some binding capacity. From the specific IgG1 response analyses it is revealed that by immunising the BN rats with 30 μg of intact BLG alone a statistically significant antibody response was reached, while immunising the rats with partially digested BLG, containing 30 μg of intact BLG together with 170 μg of BLG digestion products, a much lower antibody response was generated. This indicates that the digestion products reduced the antibody response induced by the intact BLG.

From Fig. 7 it is evident that both the high dose (200 μg) and the low dose (30 μg) of intact BLG as well as partially digested BLG had sensitising capacity being able to induce specific IgE antibodies, though only the intact BLG alone to a statistically significant level. Digested BLG and the two fractions hereof, had on the other hand, no sensitising capacity. These results reflects the results of the specific IgG1 responses, indicating that intact BLG have a stronger sensitising capacity when dosed alone, compared to being dosed in combination with peptide fragments from the very same protein.

Analysis of the specific IgA response (Fig. 8) shows that only the high (200 μg) and the low dose (30 μg) of intact BLG could induce specific IgA antibodies, both to a statistically significant level. These results indicate that the specific IgA response parallels the specific IgG1 and IgE response.

For examination of the avidity between specific antibodies raised in the different groups of rats and the intact BLG as well as the different pools of BLG-digests, a KSCN inhibition ELISA assay was performed for those individual sera for which it was possible to obtain an OD of a minimum of 0.5. No statistically significant differences were observed for the avidity between specific IgG1 and any of the tested antigens. For example there was no statistically significant difference in the avidity of the binding between specific IgG1 antibodies raised in rats immunised with intact BLG and the antigen solutions of intact BLG, partially digested BLG, digested BLG or large fragments, respectively, though there seems to be a tendency of the antibodies reacting with a lower avidity towards the digestion products compared to the intact BLG. Also no statistically significant differences in avidity were seen between specific IgG1 raised in the different groups of rats.

Discussion

The present study confirmed the majority of earlier studies showing that BLG is a protein stable to simulated human digestion, showing that BLG is virtually completely resistant to pepsinolysis, while being more susceptible to digestion by trypsin and chymotrypsin under physiologically relevant conditions [12, 24]. However, once broken down to larger fragments, BLG was rapidly digested to smaller peptide fragments of sizes less than M_r 4,500, of which more than 75% had an apparent M_r between 500 and 2,000. This suggests that when intact BLG is digested to a few larger fragments, these may not retain sufficient

structure to allow them to resist further degradation by proteases, rendering these fragments even more susceptible to additional digestion. Peptide fragments generated during the digestion process was shown to aggregate to complexes of larger sizes of up to M_r 24,500. With fractionation of the peptide fragments by means of GPC it was demonstrated that especially the peptides composed of charged amino acid residues tended to form aggregates. This is in accordance with another study, showing likewise that peptide fragments resulting from the digestion process of the peanut allergen Ara h 1, formed the largest complexes when composed mainly of charged and least of hydrophobic amino acid residues [21], indicating that non-covalently interactions other than hydrophobic are the main players in the formation of aggregates.

Food allergy develops as a result of impaired formation or break down of oral tolerance. The immune system of the BN rats used in this study has never been introduced to BLG and the rats are consequently not tolerant to this protein. The present study showed that only immunisation solutions containing intact BLG could induce specific IgG and IgE responses in the BN rats, and only intact BLG alone to a statistically significant degree. Digestion products generated by simulated gastro-duodenal digestion could on the other hand neither induce an immunogenic nor an allergenic response. This demonstrates that the digestion process was successful in abolishing the sensitisation capacity of BLG and demonstrates the importance of a functional digestive system for lowering the risk of developing food allergies. This is in agreement with studies of Untersmayr et al. [25] and Scholl et al. [26] showing that impairment of digestion enhances the sensitising capacity of caviar and hazelnut proteins in a mouse model and support the inclusion of an assessment of the stability of proteins to *in vitro* digestion by proteases as a parameter in the weight-of-evidence approach for evaluation of the potential allergenicity of novel proteins in genetically modified food as recommended by the EFSA panel in 2010 [27]. Results from the present study showed that digestion of a food allergen completely abolish its sensitising capability. This is in contrast to our previous study, showing that Ara h 1 retained both the sensitising, IgE reacting and eliciting capacity, and thereby acted as a 'complete' allergen, when digested to small peptide fragment of less than M_r 2,000 [11]. Additionally, in another study we showed that digested Ara h 1 with a peptide distribution profile very similar to that of digested BLG in the present study, likewise retained its sensitising capacity [21], proving that sizes of the break down products not alone determine whether or not the immune system mount a sensitising response. This also demonstrates the complexity of the immune mechanisms involved in directing the immune response towards a tolerogenic versus sensitising one. While the sensitising capacity of BLG was lost when digested to smaller peptide fragments, both the whole pool of digested BLG as well as the fraction of large complexes retained the binding capacity. Antibodies from BN rats immunised with the high dose of BLG could still react with the digestion products, though with a lower response than towards the intact protein, indicating that some but not all epitopes survived the digestion process, in agreement with results of Clement et al. [7]. This suggests that patients sensitised to intact BLG may still recognise the gastro-duodenal digestion products of BLG and that the digestion products may retain the capacity to elicit allergic responses. This may especially be true because of the aggregating tendency of the peptide fragments. Aggregation has earlier been suggested to be the reason for the sustained allergenic capacity of digestion products of allergens, which in theory were too small to contain two epitopes and thereby not big enough to cross-link two IgE molecules bound to the surface of mast cells or basophils [11, 21, 28]. The finding that IgE epitopes on BLG survive digestion is in accordance with previous studies, stating that the major IgE epitopes reported in the literature by Jävinen et al. [6] and Cocco et al. [5] corresponded to areas of BLG where peptides did resist simulated gastro-duodenal digestion [24]. This is in agreement with the current study and could support the presence of major linear B cell epitopes on BLG. Results from the avidity measurements, showing that no significant differences existed for the avidity of IgG1 antibody

binding to intact versus digested BLG, further strengthens the likelihood of linear epitopes. On the other hand if most epitopes of the BLG molecule were conformational, one would expect a significant reduction in the binding strength between antibodies and antigens, after the digestion process, because of the reduced possibility of small peptides to sustain sufficient structural integrity to bind antibodies raised against conformational epitopes with a similar avidity. It has been shown that a close relationship exist between the specificity of BLG specific IgE and IgG epitopes, showing that the major linear IgG epitopes generally colocalised with the major linear IgE epitopes [6]. If our results on IgG avidity are representative for IgE avidity, the present results are in accordance with earlier studies showing the very importance of just linear IgE epitopes on BLG [6, 29]. It may not be possible to generalise data on linear epitopes from BLG to other food allergens and in contrast to the above assertion we have previously shown that even small peptide fragments emerging from the digestion of Ara h 1 retained sufficient structural integrity to function as conformational IgE binding epitopes when present in aggregates probably resembling the intact Ara h 1 molecule [21, 30].

It is well recognised that pHF based on milk proteins retain residual allergenic capacity which is generally significantly higher than that of eHF [16]. On the other hand pHF may be able to induce specific tolerance while eHF fails to do this [17, 18]. This indicates that certain specific features are required for a peptide mixture to induce oral tolerance and that different peptide mixtures may influence the intestinal mucosal immune system in different ways, even when based on the same parent protein mixture. The preventive effects of various hydrolysates have been shown not solely to depend on the degree of hydrolysis [31]. Does the digested BLG function as the pHF being able to induce tolerance mechanisms or does it work like eHF by failing to achieve such tolerance? To examine if the digested BLG induce tolerance mechanisms or if it was simply ignored by the immune system, specific serum IgA was measured, since specific serum IgA have been suggested to be an indicator for development of tolerance, correlating inversely with specific serum IgE in allergen sensitised patients [32, 33]. However, specific IgA levels in BN rats seem to correlate with the specific IgG1 and IgE responses. These results are therefore not informative about whether the digested BLG inheres tolerogenic potential or whether the digestion products were not recognised by the immune system of the rats.

The present study showed that antibody levels in rats immunised with partially digested BLG (containing 30 µg of intact BLG together with 170 µg of BLG digestion products) were strongly reduced compared to the antibody level in rats immunised with the low dose of intact BLG. The amount of intact BLG was the same in the two immunisation solutions, the difference being the additional presence of digestion products in the partially digested BLG. This indicates that the reduced response was induced by the BLG derived peptide fragments. As we see it, the lower response could be a result of two different situations. The first involves the masking of epitopes implicated in the sensitisation phase, by means of the peptide fragments camouflaging the epitopes by an aggregate formation with the intact molecule. The second situation involves tolerogenic properties of the peptide fragments, which by co-immunisation with the intact protein leads to impaired sensitising capacity of the intact BLG dimeric molecule. The first situation however seems unlikely, based on the knowledge from the analytical GPC showing that the dimeric BLG molecules did not form complexes with the peptide fragments present in the partially digested BLG, or at least not to a level which could be detected. This point to the second situation, that co-administration of the intact and digested BLG induced tolerance mechanisms, leading to antibody responses of significantly lower levels when administrating intact BLG together with peptide fragments from the same protein, compared to administrating the intact BLG alone.

Tolerance may be induced by fundamentally different mechanisms, shown in animal studies of low- versus high-dose induced tolerance [20] and in studies of carrier-specific tolerance induction [34]. From a study of a ovalbumin derived peptide and analogous hereof it was shown that even small differences in peptide composition can make huge differences in deciding if the peptide strengthens or weakens the allergen-specific sensitisation response [35].
Whether the peptide fragments are able to induce tolerance mechanisms alone, or whether they need to be presented to the immune system together with the native BLG, remains to be demonstrated. If so, it also remains to be demonstrated if the tolerance mechanisms involved are the same. Peptides may be processed and presented differently from the intact protein, and there is the possibility of presenting a greater variety of different T cell as well as B cell epitopes from the intact than the digested protein. Maybe the use of intact protein plus peptides ensures that there will be achieved tolerance against not only those sequences presented by the peptides but also other sequences of the protein and thereby the entire protein.

Further studies are needed in order to reveal if digested BLG alone have tolerogenic capacity, studying the mechanisms underlying tolerance induction, and to elucidate the general tolerogenic capacity of co-administration of intact allergen with peptide fragments hereof. This would in addition contribute to a better understanding of the underlying mechanisms of sensitisation versus tolerance induction which could be useful in the future for new strategies in the prevention and treatment of allergic diseases. In agreement with the message by Fritsche et al. [17], animal models would in such situations certainly be helpful in prediction of allergenic versus tolerogenic potential of different hydrolysates and in the understanding of the mechanisms involved.

Abbreviations

BLG, *β*-lactoglobulin; BN, Brown Norway; CMA, cow's milk allergy; eHF, extensively hydrolysed formula; ELISA, enzyme-linked immunosorbent assay; GPC, gel permeation chromatography; IC_{50}, inhibition concentration at 50% reduction of response; KSCN, potassium thiocyanate; MALDI-TOF, matrix-assisted laser desorption/ionisation time-of-flight; MS, mass spectrometry; PBS-T, PBS containing 0.01% Tween 20; pHF, partially hydrolysed formula; RP-HPLC, reverse phase high-performance liquid chromatography; RT, room temperature; TMB, 3,3',5,5-tetramethylbenzidine.

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Figure Legend

Figure 1 Prep grade GPC of BLG digests. To separate intact BLG from the digestion products generated after 120 min of simulated gastric digestion plus 15 min of simulated duodenal digestion and for fractionation of the digestion products, preparative GPC was performed. Based on the 280 nm absorbance GPC profile, it was decided to make four different pools of BLG-digests, indicated with the curly brackets; partially digested BLG, digested BLG, large complexes and small complexes. Partially digested BLG contained all

products resulting from the digestion process, including approximately 15% intact BLG, that resisted the digestion. Digested BLG consisted of all digestion products, after dissociation from the intact BLG, while the large complexes and small complexes were different fractions hereof separation based on the sizes of aggregates, together corresponding to the digested BLG.

Figure 2 Analytical RP-HPLC profiles for intact BLG and BLG digests. Comparison of chromatography profiles, shown with absorbance at 220 and 280 nm, for intact BLG (**A**), gastric digests of BLG (**B**), partially digested BLG after gastro-duodenal digestion (**C**), digested BLG (**D**), large complexes (**E**) and small complexes (**F**).

Figure 3 Amino acid frequency distribution of intact BLG and BLG digests. Comparison of amino acid distribution profiles for intact BLG (**A**), partially digested BLG (**B**), digested BLG (**C**), large complexes (**D**) and small complexes (**E**). The bars represent the frequency percentage for each amino acid(s), represented by their one letter code, grouped according to their physico-chemical features.

Figure 4 Peptide mass distribution for digested BLG and fractions hereof. Mass distribution profiles for digested BLG (**A**), large complexes (**B**) and small complexes (**C**), shown in histograms, where each bar corresponds to a peptide mass interval of M_r 500.

Figure 5 Analytical GPC for intact BLG and BLG digests. Comparison of chromatography profiles, shown with absorbance at 220 and 280 nm, for intact BLG (**A**), partially digested BLG (**B**), digested BLG (**C**), large complexes (**D**) and small complexes (**E**).

Figure 6 Specific IgG1 responses. Comparison of specific IgG1 titre (Log_2) values, for groups of rats immunised with either PBS (control), 200 μg of intact BLG, 30 μg of intact BLG, 200 μg of partially digested BLG (containing 30 μg of intact BLG), 200 μg of digested BLG, 200 μg of large complexes or 200 μg of small complexes. Sera from all rats in each immunisation group were examined for specific IgG1 antibodies against intact BLG, partially digested BLG, digested BLG, large complexes and small complexes, shown with different symbols. Each symbol represents an individual rat. Horizontal bars indicate the median IgG1 titre value for each group of rats towards the given antigen product. Statistically significant differences between groups of rats were determined by use of Kruskal-Wallis test followed by Dunn's multiple comparison test. Asterisks indicate statistically significant differences of the given group compared to the control group.

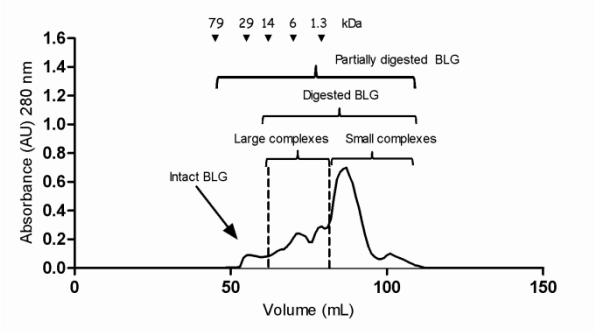
Figure 7 Specific IgE response against intact BLG. Comparison of BLG-specific IgE titre (Log_2) values for groups of rats, immunised with either PBS (control), 200 μg of intact BLG, 30 μg of intact BLG, 200 μg of partially digested BLG (containing 30 μg of intact BLG), 200 μg of digested BLG, 200 μg of large complexes or 200 μg of small complexes. Each symbol represents the IgE titre value for an individual rat. Horizontal bars indicate median IgE titre value for each group of rats. Statistically significant differences between groups of rats were determined by use of Kruskal-Wallis test followed by Dunn's multiple comparison test. Asterisks indicate statistically significant differences of the given group compared to the control group.

Figure 8 Specific IgA response against intact BLG. Comparison of BLG-specific IgA titre (Log_2) values for groups of rats, immunised with either PBS (control), 200 μg of intact BLG, 30 μg of intact BLG, 200 μg of partially digested BLG (containing 30 μg of intact BLG), 200 μg of digested BLG, 200 μg of large complexes or 200 μg of small complexes. Each symbol represents the IgA titre value for an individual rat. Horizontal

bars indicate median IgA titre value for each group of rats. Statistically significant differences between groups of rats were determined by use of Kruskal-Wallis test followed by Dunn's multiple comparison test. Asterisks indicate statistically significant differences of the given group compared to the control group.

Figure 9 Avidity measurements of specific IgG1 antibodies. Comparison of specific IgG1 antibody binding avidities towards the given antigen product, expressed as the KSCN concentration needed for inhibition of 50% response, for groups of rats immunised with either 200 µg of intact BLG, 30 µg of intact BLG or 200 µg of partially digested BLG. Sera from all rats mounting a specific IgG1 response of at least OD 0.5 were included in the examination of IgG1 avidity. Each symbol represents the IgG1 avidity for an individual rat. Horizontal bars indicate mean IgG1 avidity for each group of rats. Statistically significant differences between groups of rats were determined by use a parametric One-way ANOVA, followed by the Tukey's multiple comparison test for comparison of three or more groups.

Figures



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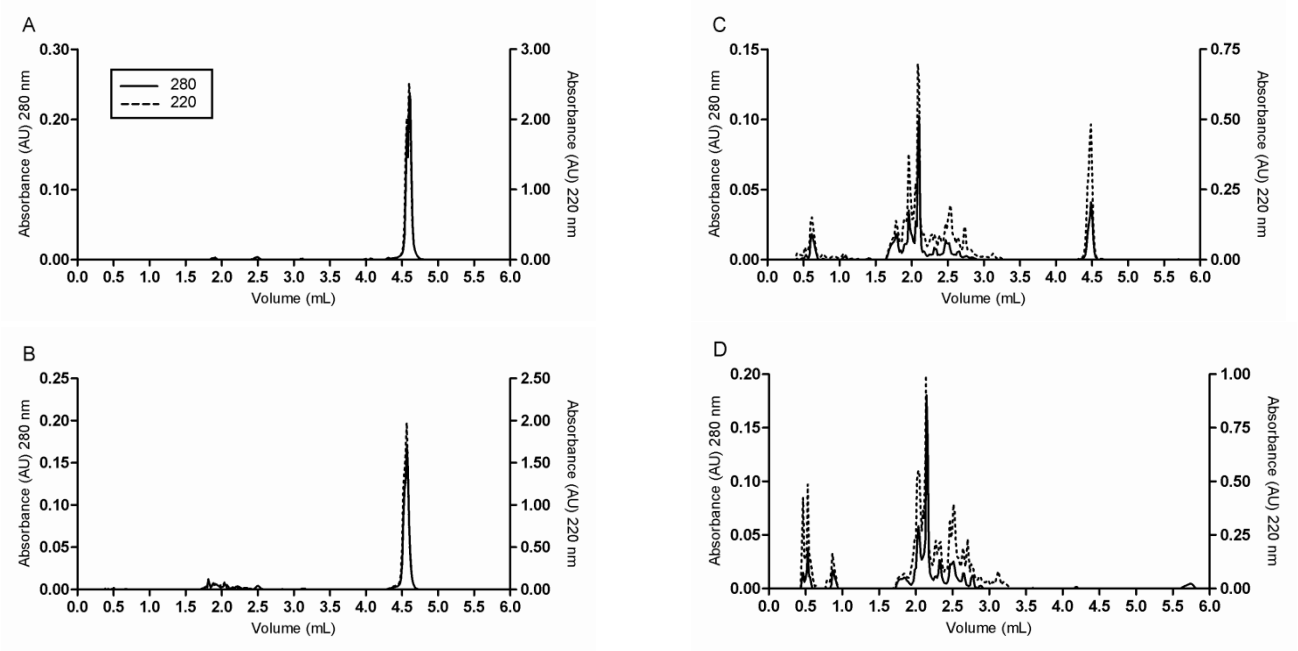

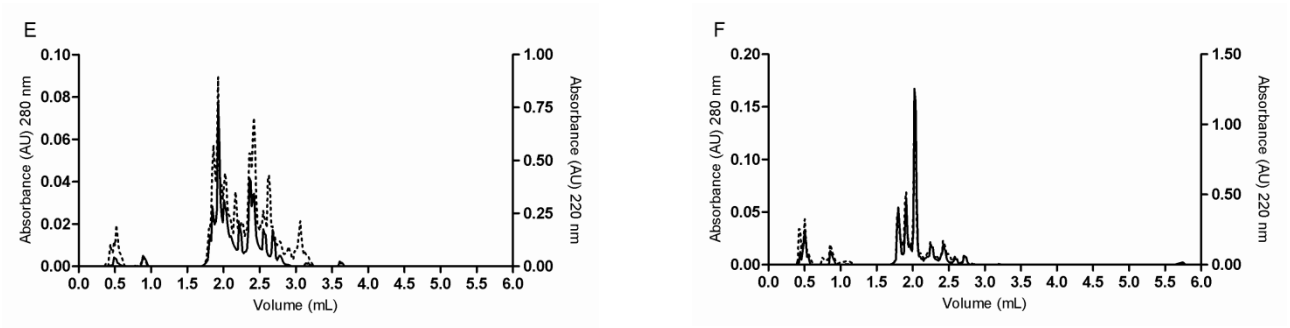
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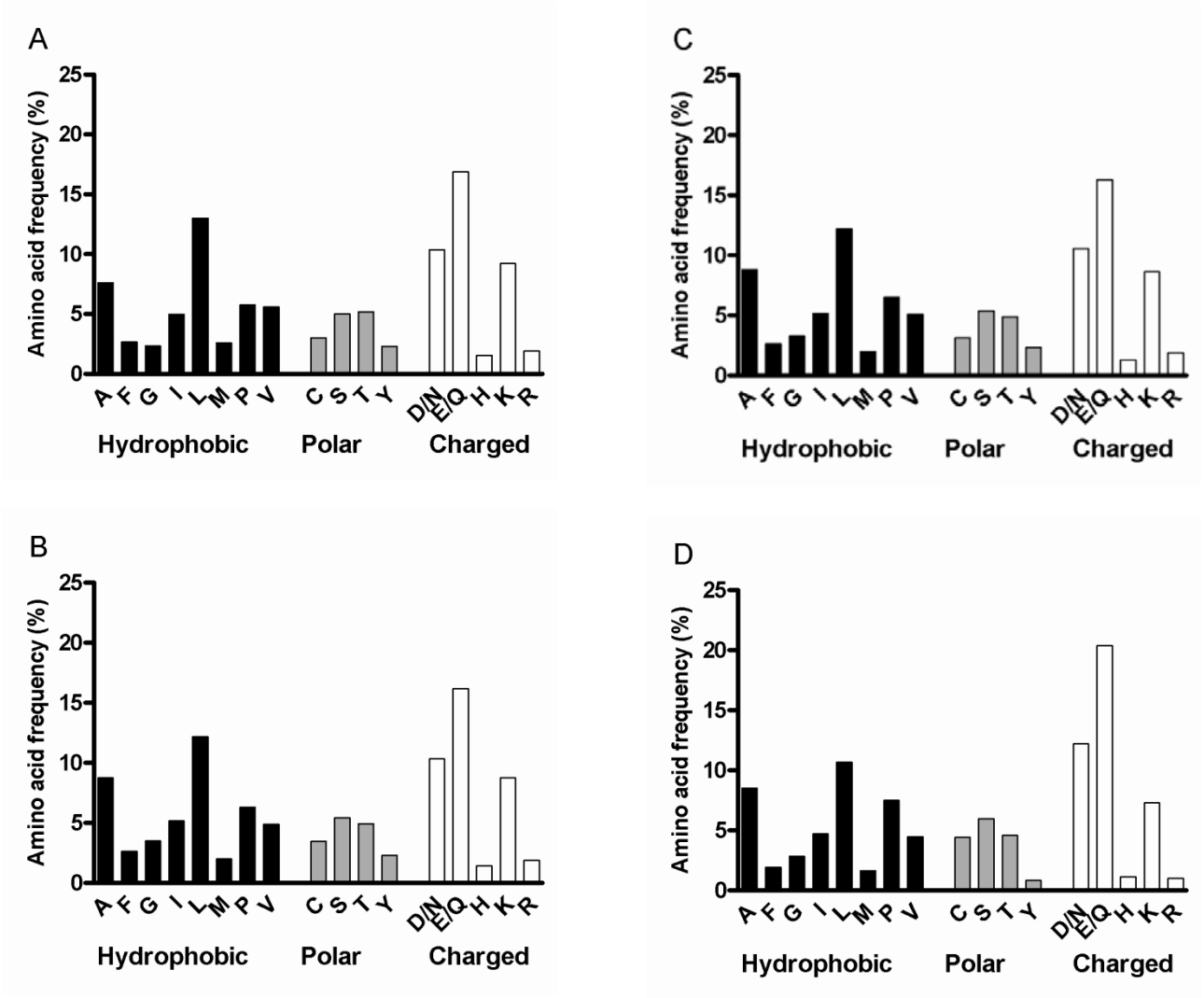
Figure 2.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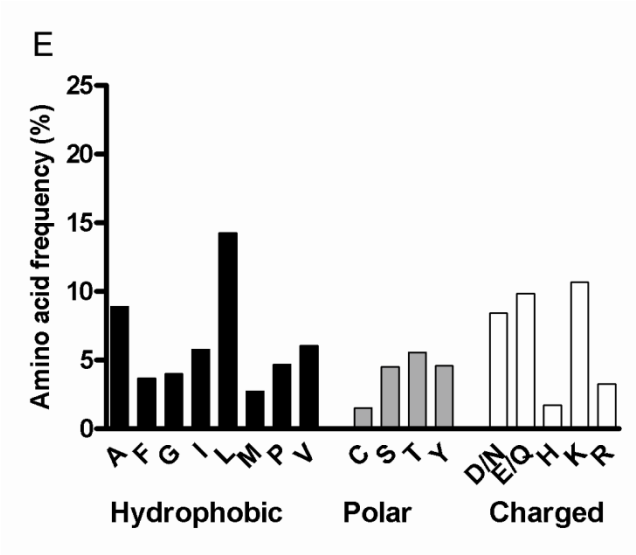

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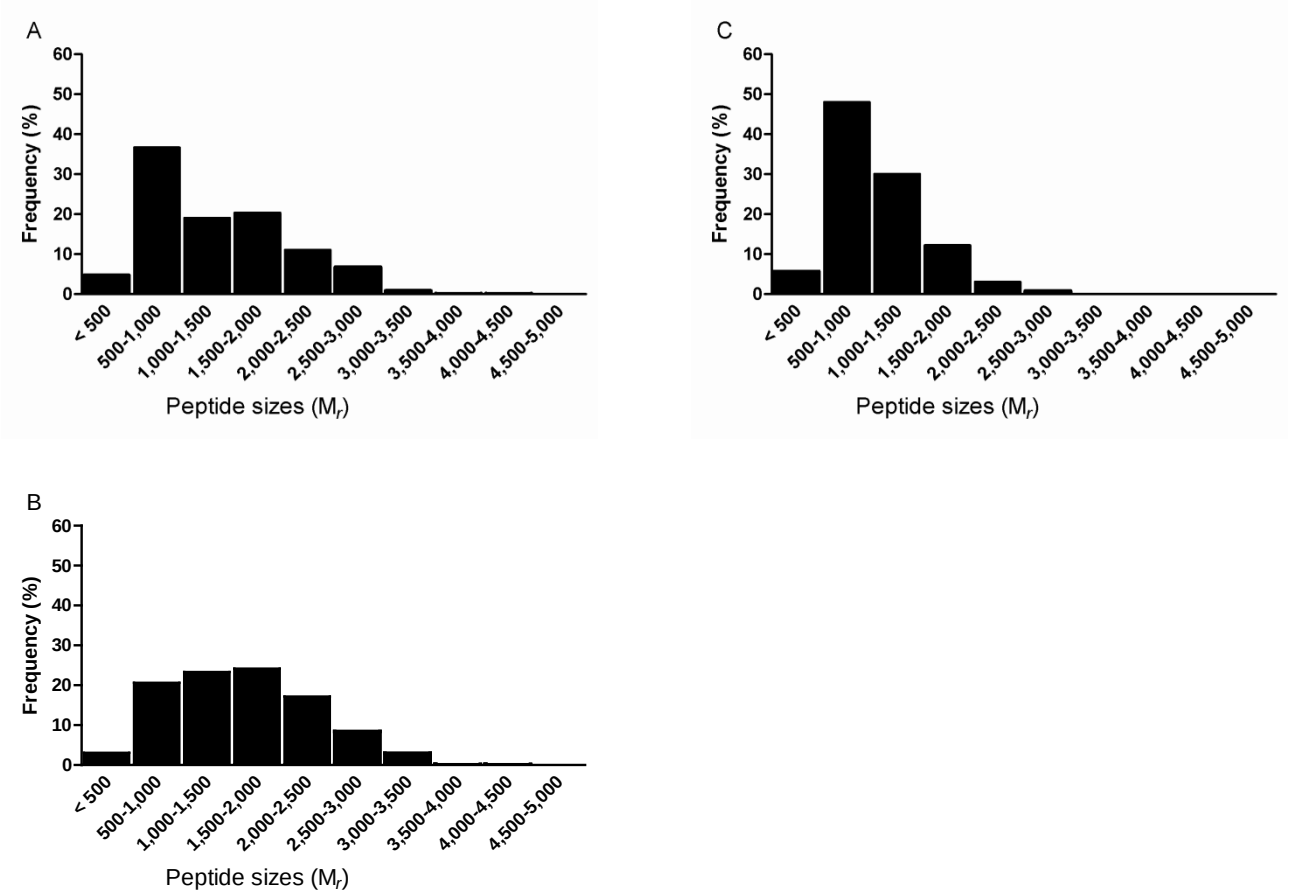

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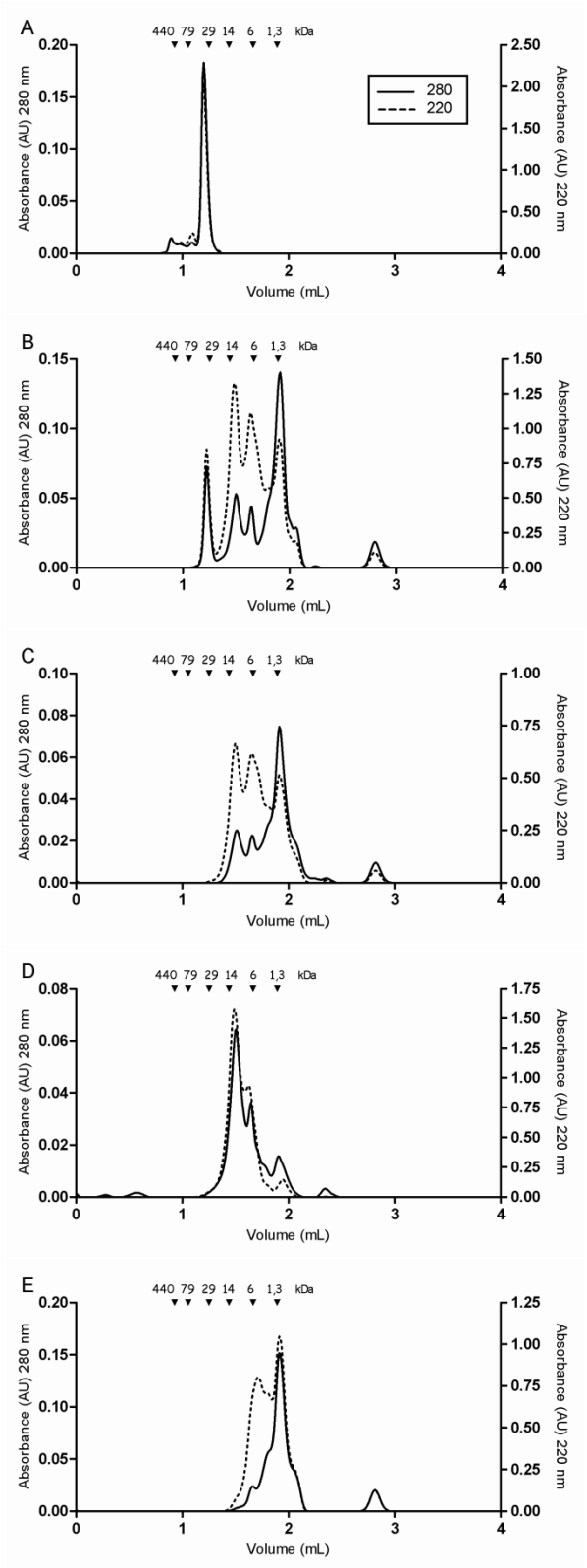
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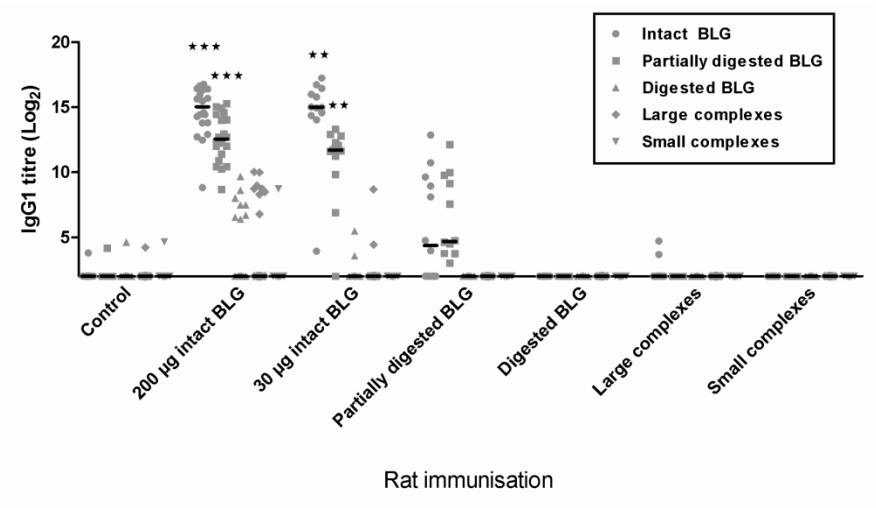
Figure 5.



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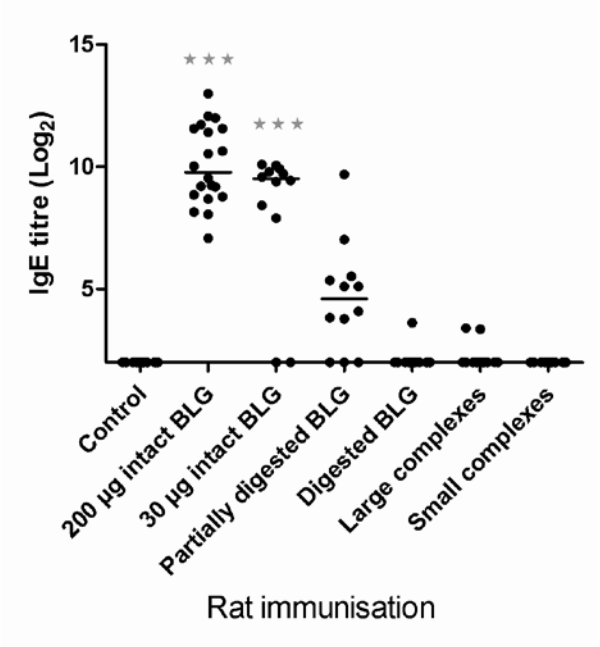

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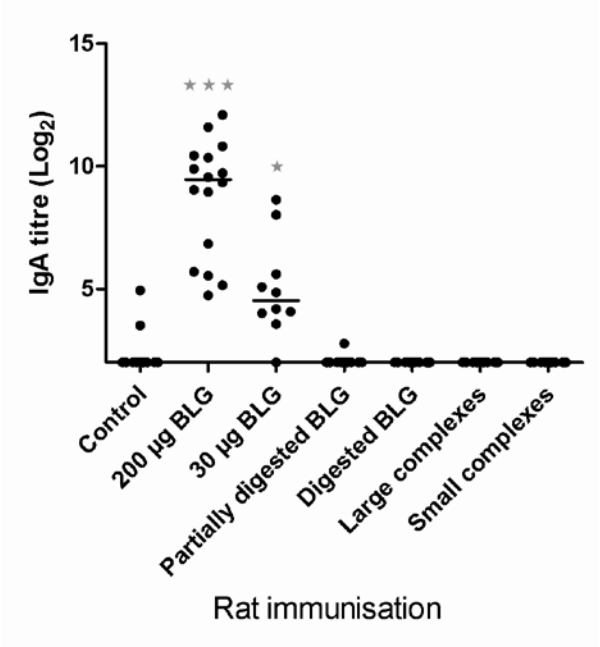

Figure 8.

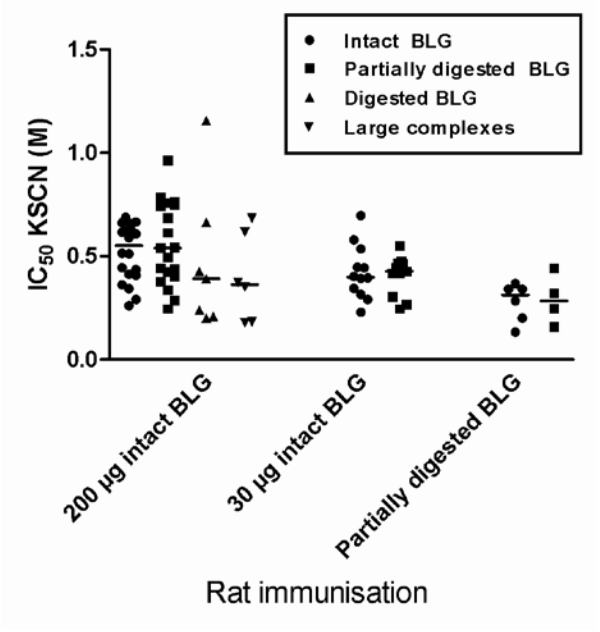

Figure 9.

SUMMARISING DISCUSSION

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Novel proteins in genetically modified foods have since the eighties been introduced into our food supply, and probably will continue to [1]. The potential allergenicity of these 'new' proteins is of major concern when bringing genetically modified foods into the market. Strategies for safety assessment of the potential allergenicity have been recommended, based on either a decision tree [2] or a weight-of-evidence approach [3;4]. A complete proof of safety seems, however, impossible, because of the lack of knowledge about what makes a dietary protein a food allergen. An evaluation of the stability to digestion with pepsin remains a central part of the allergenicity safety assessment of a novel protein, despite the fact that the relationship between resistance to digestion and allergenicity has been questioned in recent years [5-7].
Undoubtedly, survival in the GI tract plays an important role for the sensitising capacity of some food allergens. This has been demonstrated in the current thesis, where the cow's milk allergen BLG was shown to lose its sensitising capacity when broken down to peptides in an *in vitro* model simulating the human gastro-duodenal digestion process. While the peptide fragments of sizes less than 4.5 kDa, generated during the simulated gastro-duodenal digestion process of intact BLG, failed to induce specific IgE antibodies in BN rats, small peptides fragments of sizes less than 2 kDa generated during simulated gastro-duodenal digestion of the peanut allergen Ara h 1 did on the other hand retain the capacity to induce specific IgE antibodies in the same BN rat model. This demonstrates that while degradation may be a successful method for abolishing the sensitising capacity of some food allergens, it is not a general phenomenon applicable for all food allergens. In parallel to intact dietary proteins, where some proteins may have an intrinsic capacity to sensitise an individual while others may not, peptide mixtures derived from the digestion of food allergens may also possess differences in their ability to sensitise; an ability not only depending on the sizes of the peptides.

The peptide fragments of sizes less than 2 kDa, generated during the *in vitro* gastro-duodenal digestion of Ara h 1, could not only sensitise but also bind IgE and elicit a degranulation response, thus possessing all properties that define a 'complete' food allergen. These findings seem surprising, since the peptide fragments are smaller than originally thought to be the lower limit for peptides with inherent allergenicity [2;8-10]. Results presented in this thesis demonstrate that there does not exists an absolute correlation between resistance to digestion and allergenic potential, and that establishing a MW size below which peptide fragments from food allergens are not allergenic could be very challenging if not impossible. Lability to digestion may certainly be regarded as a factor that reduces the risk associated with allergenicity [1] but this project emphasises that protein stability to peptic proteolysis should be assessed along with other test parameters and that potential allergenicity of novel proteins should be evaluated on a case-by-case basis.

Peptide fragments generated during both the gastric and gastro-duodenal digestion of the purified Ara h 1 were shown to aggregate to complexes of larger sizes. Whether an aggregation of the small peptides to complexes of larger sizes was the reason for the observed sensitising capacity was investigated by subjecting the digestion products to fractionation according to the sizes of the peptides and the aggregates formed. By means of this fractionation step, the digestion products of Ara h 1 lost their sensitising capacity. Both the peptide fragments in the whole pool of digested Ara h 1 as well as in the fractions of digested Ara h 1, were shown to aggregate, though to different degrees. This demonstrates that if the sensitising capacity of digested Ara h 1 is a result of the peptide fragments aggregating to complexes of larger sizes, this is not simply a result of them aggregating but more a result of how they aggregate. This however, did

neither confirm nor reject if the sensitising capacity was indeed a result of the peptide fragments aggregating to larger complexes. Instead it created the basis for the hypothesis that either the sensitising capacity was a result of the peptide fragments being in aggregated complexes resembling the intact Ara h 1 or that the complete mixture of free peptide fragments had an intrinsic capacity to sensitise by themselves. That even very small peptides may hold the capacity to induce specific antibodies is evident from research with small free synthetic peptides [11-13]. In addition, the importance of formation of aggregates by small peptides has also been acknowledged [3;14-19]. It was for example suggested that assembling of small peptides in some kind of scaffold could enhance the immunogenicity of the small peptides, because the structural stability could be of great importance [17]. That the allergenic capacity of peptide fragments formed by the digestion process of food allergens may be a result of formation of larger complexes, have earlier been suggested for Ara h 1 [14], for the Brazil nut allergen Ber e 1 [19] as well as for digestion products of a cow's milk allergens [15;18]. In light of the theoretically importance for antibody cross-linking events on APC, B cells, mast cells as well as basophils; an aggregation of the small peptide fragments could hypothetically explain the very potent sensitising and eliciting capacity of the digested Ara h 1 presented in the current thesis. Aggregation of peptide fragments creates the possibility of IgE binding epitopes being in close vicinity. Thus cross-linking of either surface-bound IgE antibodies on APC, surface-expressed IgE antibodies on B cells or FcεRI-bound IgE on the surface of mast cells or basophils could occur (Figure 1).

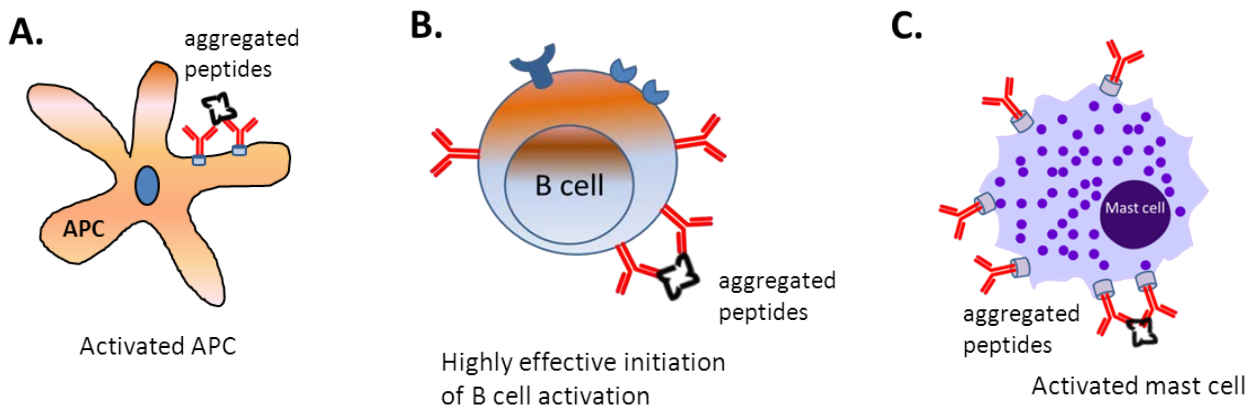

Figure 1. A hypothetical explanation for the importance of aggregate formation of small peptide fragments generated during the digestion process. Aggregation of small peptides (too small to be allergenic by themselves), may explain the potential to both induce sensitisation and eliciting allergic responses. Aggregation of small peptides allows epitopes to be in close vicinity permitting cross-linking events of; **A.** Surface-bound antibodies on APC, **B.** Surface-expressed antibodies on B cells, and **C.** Surface-bound antibodies on mast cells or basophils.

The epitope mapping study of intact and digested Ara h 1 indicated that IgE binding epitopes of Ara h 1 survived the digestion process. This was most pronounced in the findings that five epitope motifs, corresponding to more than 65% of all epitopes identified for the five peanut allergic patients, were identified for both intact and digested Ara h 1. Furthermore, realising that all identified epitope motifs could be categorised as conformational supports the assumption that digested Ara h 1 is in an aggregated conformation resembling the intact molecule. For the first time it has been demonstrated, that even though a protein is digested to very small peptide fragments, the survival of conformational epitopes may be possible.

For a dietary protein to sensitise through the intestinal mucosal immune system epitopes need to survive the digestion process or new ones need to be generated. IgE specific for epitopes surviving the digestion

process has been hypothesised to be associated with a severe and persistent food allergic phenotype [20-22]. The epitope mapping results presented in the current thesis may support this hypothesis, as the peanut allergic patients had a history of both severe and persistent peanut allergy. Further this study supports the idea that IgE binding epitopes resisting the digestion process may be used as predictive biomarkers for the allergic phenotype [20]. In contrast to earlier studies suggesting a particular important role for linear IgE binding epitopes in food allergy [23], the present study suggests in addition that recognition of conformational IgE binding epitopes could be of major importance.
While conformational epitopes may be of great importance for the peanut allergen Ara h 1, the cow's milk allergen BLG-specific IgE binding epitopes surviving the digestion process seem to be linear.

To avoid the introduction of novel dietary proteins with sensitising capacity, a clear guidance on how to interpret the result from a digestibility assay seems advisable, especially in light of the results presented here. This is in agreement with Fu [5] stating that a consensus on how to relate the measured digestibility to the allergenic potential of novel proteins needs to be reached, in order to allow susceptibility to digestion as a predictive tool. First, the choice of an applicable *in vitro* digestibility assay, preferable simulating physiologically relevant gastric as well as duodenal human digestion process, seems highly relevant. Second, the choice of appropriate methods, which should be performed in addition to SDS-PAGE, allowing for evaluation of the susceptibility to digestion of the novel protein, seems advisable. These methods should compensate for the inadequacies of the SDS-PAGE and allow for detection of even small amounts of residual intact protein, for detection of even very small peptide fragments as well as for detection of aggregates. Third, an assessment of the stability of the peptide fragments generated during the digestion process should be evaluated on equal terms as the intact protein. Fourth, an assessment of the susceptibility to digestion should be combined with different immunologically assays, allowing for detection of residual IgE binding capacity, eliciting properties as well as sensitising properties based on studies in animal models. Some of these suggestions are issues mentioned by the EFSA panel in 2010 [3].

While the digestion products of Ara h 1 retained sensitising capacity, digestion product of BLG failed to induce any specific antibodies. Instead the results suggested that the digestion products from BLG possessed tolerogenic capacity. This was indicated by the significantly lower sensitising potential of intact BLG co-administered with digested BLG compared to the sensitising potential of same amount of intact BLG alone. Whether the potential tolerogenic capacity was a feature of the peptide fragments *per se* or if the tolerogenic capacity was a result of the co-administration with intact BLG could not be elucidated with the study design used.
These studies with digestion products from two different food allergens revealed that mixtures of peptide fragments, with very similar peptide size distributions may not necessarily stimulate the immune system in similar ways, but instead may direct the immune system in different directions. Collectively these studies show that several properties of dietary proteins will contribute to the allergenic sensitising potential, characteristics that involve not only the sizes of the peptides generated during the digestion but also the structure of these peptides, probably in addition to several other features. This is in agreement with Wijk and Knippels [24] reporting that several characteristics may together determine whether a protein will meet the requirements for inducing an immune response or not.
Theoretically the observed immunologically differences between digested Ara h 1 and digested BLG could explain the variations seen between the allergic phenotypes associated with peanut and cow's milk allergy. The potent allergenic capacity of digested Ara h 1 may explain why peanut allergy is such a persistent and severe type of food allergy compared to cow's milk allergy [25-29].

In conclusion this thesis demonstrates that resistance to digestion does not necessarily correlate with allergenicity and that peptide fragments generated during the digestion process, too small to be allergenic by themselves, may aggregate to complexes of sufficient size and with sufficient structure to retain sensitising potential. The combined results presented in this PhD thesis showed the complexity of the mechanisms underlying sensitisation and demonstrated that defining a single or even several characteristics of a dietary protein identifying it as a food allergen is very difficult if not impossible.

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